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REVIEW ARTICLE

Microfluidic nanochips for rapid exosome isolation and multiplex biomarker profiling from a single blood drop: Toward sample-to-answer liquid biopsy

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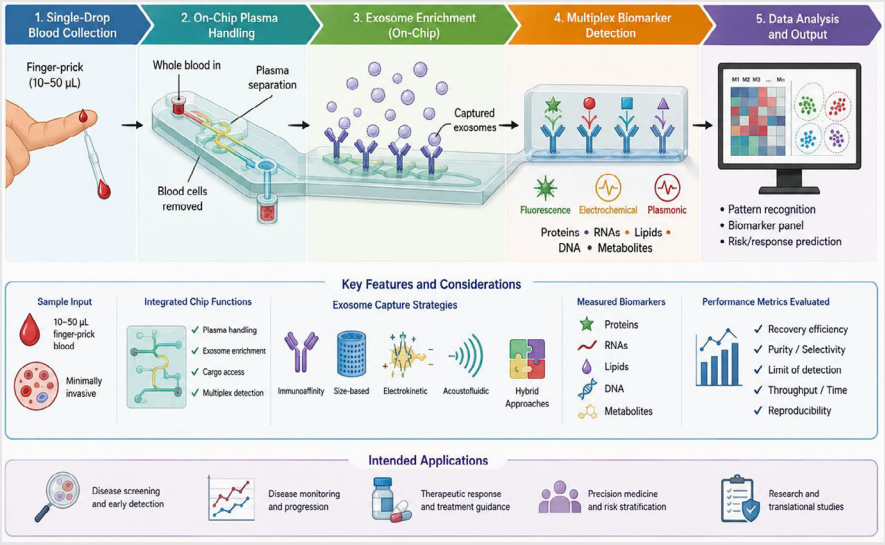
Graphical Abstract



A microfluidic nanodevice that analyses a single volume droplet of whole blood (10–50 microlitres (µL)) forms a fast and fully integrated exosome-derived liquid biopsy. The closed system architecture involves plasma separation on chip, exosome enrichment by immunoaffinity and various size based, electrokinetic, acoustofluidic or hybridized strategies, and controlled cargo access followed by multiplex biomarker interrogation by various optical, electrochemical, or plasmonic transduction strategies. Sophisticated nano-biointerface designs give high capture efficiency and at the same time prevent fouling and shear situation damage. The platform overcomes some of the fundamental hurdles of whole blood assays such as haemolysis, viscosity effects, anticoagulant interference, specific platelet activation, etc., using normalized metrology and classification through algorithm to produce clinically practicable results. This sample-to-answer approach supports scalable applications in disease screening, therapeutic

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monitoring, and relapse detection, advancing reliable exosome diagnostics from a single blood drop.

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Abstract

Background: Exosomes and other small extracellular vesicles carry molecular cargo that can reflect disease status, therapeutic response, and relapse dynamics. Currently, most exosome-analysis workflows are centralized, require milliliter-sized blood samples, involve extended isolation procedures, and depend on separate downstream assays. Single-drop microfluidic nanochips, which can process finger-prick blood volumes of approximately 1050 μL within a closed cartridge, offer a promising route toward integrated sample-to-answer liquid biopsy platforms.

Main Body: These systems may integrate plasma handling, exosome enrichment, cargo access, multiplex biomarker detection, and algorithmic classification. This review highlights key on-chip enrichment techniques, including immunoaffinity, size selection, electrokinetic, acoustofluidic, and hybrid methods. It also discusses nano-biointerface designs that improve vesicle capture efficiency while reducing fouling and shear-induced vesicle damage. In addition, multiplex sensing architectures for optical, electrochemical, and plasmonic

transduction are explored, with particular attention to challenges associated with whole-blood measurements, including anticoagulation, sample variability, platelet activation, viscosity, and hemolysis.

Conclusions: Single-drop exosome nanochips have the potential to transform liquid biopsy by enabling rapid, low-volume, integrated analysis of extracellular vesicles. However, successful clinical translation will require clear validation requirements, standardized reporting expectations, and defined use-case roadmaps for screening, disease monitoring, and therapeutic-response assessment. Establishing realistic performance standards and robust engineering parameters will be essential for making single-lab exosome nanochip platforms reproducible and clinically applicable.

KEYWORDS

exosomes, extracellular vesicles, liquid biopsy, microfluidics, multiplex biosensing, nanochips, point-of-care diagnostics

Key points

- Single-drop microfluidic nanochips enable rapid EV/exosome enrichment from finger-prick blood. Integrated cartridges combine plasma handling, vesicle capture, cargo access, and multiplex biomarker detection.
- Orthogonal controls are essential to reduce lipoprotein, hemolysis, and platelet-derived interference.
- Clinical translation requires standardized validation, reproducibility, and manufacturable sample-to-answer workflows.

1 | INTRODUCTION

Exosomes are nanoscale extracellular vesicles that carry proteins, nucleic acids and lipids, thereby reflecting the physiological state and cellular origin of their parent cells.[1,2] Such cargo in principle makes exosomes appealing to liquid biopsy, as single measurement can probe many molecular layers, and with proper normalization can be used to monitor longitudinally and not at a single point of detection.[3,4] Although considered significant subjects, most exosome studies are still centralized to selected labs and large sample volumes are often required. A traditional workflow will require the step of venipuncture, plasma preparation, successive isolation using ultracentrifugation or precipitation, size exclusion or affinity capture and protein and RNA measurements via separate assays.[5] All these phases cause a delay over time, dead space, operative errors and the possibility of lipoprotein, platelet and protein crystallite contamination.[5] Time-to-answer and infrastructural requirements in a clinical setting where responsibilities and choices must be undertaken quickly are usually prohibitive. Single-drop microfluidic nanochips reframe exosome testing as an integrated sample-to-answer system (around 10–50 μL by volume) has to be subjected to an enclosed cartridge, which carries out the functions of plasma processing, exosome enrichment, cargo access, multiplex sensing and algorithmic classification.[6,7] Figure 1 presents a conceptual single-drop exosome-testing workflow and links each step to representative experimental approaches reported in the literature. These include capillary micro sampling and microfluidic blood handling, point-of-care immunoaffinity capture of exosomes, nanostructured microfluidic platforms for enhanced vesicle capture, on-chip plasma or whole-blood pre-processing, and downstream classification methods that convert multiplex biomarker signals into clinically interpretable results.[8]

This type of setup would move liquid biopsy closer to the patient, enable near-real-time monitoring of a therapeutic responses, decentralize cohort studies and constant sampling with minimal patient discomfort. However, miniaturizing testing protocols down to the millilitre scale is not a trivial engineering challenge. At the microlitre scale, dead volumes are similar to the sample and nonspecific adsorption can eliminate a significant proportion

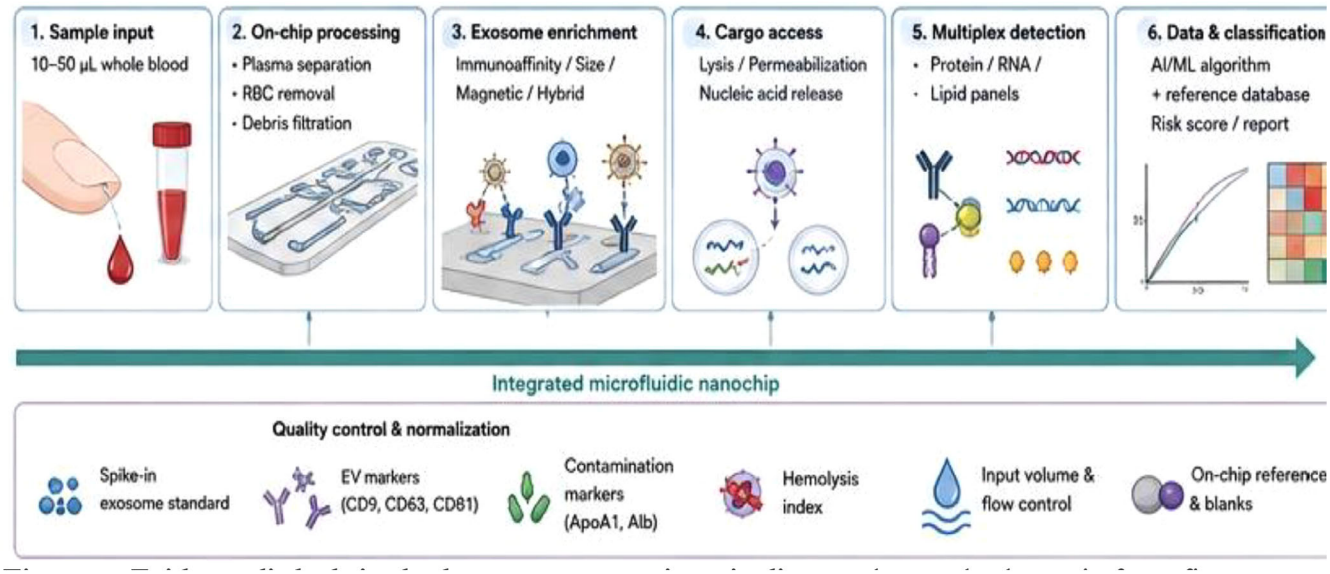


FIGURE 1 Evidence-linked single-drop exosome testing pipeline. End-to-end schematic from finger-prick blood collection to interpretive output, showing plasma handling, exosome enrichment, cargo access, multiplex detection, quality-control checks and algorithmic classification.

of the analyte, and that, although, down to the microlitre scale, little perturbation in red blood cells can cause large changes in flow rates and separation performance.[7] In the same way as in pre-analytical variables like haemolysis, platelet activation and the choice of anticoagulants might be varying the signal unless they are controlled through appropriate device design and standardized operating procedures.[7] To this end, a single-drop exosome nano-chips will need to focus on applied metrics and failure modes and analytical sensitivity alone.[9] Platforms with clinical significance are required to report recovery, purity, throughput and clogging risk, throughput and risk of clogging and at least partially compatible with subsequent protein and RNA readouts.[10] They need to incorporate controls which define the true exosome signals and confounders and they need to have reproducibility amongst chips, manufacturing batch and location.[10]

There is a difference in the interpretation of analytical sensitivity and clinical utility. Analytical sensitivity is the ability of a platform to detect allowed assay conditions at very low levels of exosomes or exosomal biomarkers, and is frequently described by limit of detection, limit of quantification or signal-to-noise ratio.[11] The term clinical utility is used to indicate whether the test result has an influence on the diagnosis, monitoring, assessment of therapeutic response or decision making process in a real patient population.[12] Thus, a platform with good analytical sensitivity in buffer or spiked plasma may lack clinical utility when applied to whole blood, reproducibility or ability to control for confounders, or offer information with no clinical endpoint. On the other hand, if there are sufficient arguments that this moderately sensitive assay reliably measures the patient-specific changes over time, then it may be useful for longitudinal monitoring.

In this review, a mechanism-based map of on-chip exosome enrichment approaches and their trade-offs is introduced, and the approaches are subsequently associated with nanoscale bio-interface engineering, which enhances capture kinetics with limited fouling and shear. We cover designs of integrated lysis and cargo access architectures, multiplex sensing architectures and whole-blood handling designs that maintain the integrity of vesicles as well as lessen the effects of haemolysis. Importantly, the review also covers magnetic and centrifugal microfluidic separation strategies, which are now widely used in exosome research and translational cartridge development. Magnetic separation uses antibody, aptamer, or ligand-functionalized magnetic beads to capture exosomes expressing canonical EV markers or disease-associated antigens, followed by magnetic concentration, washing and direct downstream sensing.[13] This approach is attractive because it is highly specific, compatible with small sample volumes, and readily integrated with fluorescence, electrochemical, or molecular readouts. However, it may introduce marker-selection bias, bead aggregation, nonspecific adsorption and incomplete release of captured vesicles. Centrifugal microfluidic separation, including lab-on-disc and centrifugal-pneumatic cartridge systems, uses rotation-driven fluid handling, sedimentation, filtration, valving and chamber-to-chamber transfer to automate plasma separation, size selection, immunocapture, washing and lysis. These systems are particularly

useful for closed, sample-to-answer operation because they reduce manual pipetting and can integrate multiple unit operations. Their limitations include hardware dependence, disc fabrication complexity, balancing requirements, possible shear or clogging effects and the need for rigorous lot-to-lot validation. Therefore, magnetic and centrifugal microfluidic approaches should be considered alongside immunoaffinity, size-based, electrokinetic, acoustofluidic and hybrid strategies when selecting an exosome-enrichment method for single-drop liquid biopsy.

Recent advances in EV-based molecular diagnostics have also expanded beyond conventional immunocapture and bulk vesicle analysis toward highly sensitive digital, molecular, and functional readouts. Emerging strategies include digital PCR-linked EV assays, DNA-barcoded antibody platforms, CRISPR-assisted EV-RNA detection, single-molecule immunoassays and bio-orthogonal click chemistry-enabled EV enrichment.[14] In particular, click chemistry-based approaches can improve EV capture efficiency, reduce nonspecific binding, preserve vesicle integrity and enable integration with downstream genetic, proteomic, and functional liquid-biopsy assays.[14] These developments highlight the need for single-drop nanochip platforms that combine efficient EV enrichment with sensitive molecular readouts and clinically interpretable outputs.

For clinical translation, analytical sensitivity and clinical utility can be separated, as there can be a modestly sensitive test, which is still useful in that they can give consistent trends to be followed. They should add a platform internal standard that spans the entire workflow where possible to be able to estimate recovery and inhibition on each run. The finger-prick sampling creates variability with the user, and therefore strong cartridges frequently incorporate some buffering mode of time variation, like passive metering, clot traps, or buffer mixing zones.[15] Engineering wise, the most typical modes of failure include mechanical and fluidic and not insidious biomolecular effects which include bubbles, clogs, incomplete priming and uneven partitioning of the flow. Lastly, the performance of manufacturing choices with regard to thermoplastic choice, surface activation, and reagent storage can be altered at the baseline, then initial prototyping must expect scaled materials and manufacturing. All in all, single-drop testing of exosomes can be credibly done using strong, integrated designs that have transparent control strategies and standardized metrology, to support decentralized clinical decision-making.

In view of the promise for translation, this review assesses microfluidic exosome technologies based on their recovery, purity, high throughputs, compatibility with platelet-rich blood, down-stream molecular assays, manufacturability and reproducibility evidence. Such framework is required since these are buffer/plasma matrix samples with high analytical sensitivity, which may not correlate with the robustness in the clinical matrices of finger-prick whole blood. Thus, across the review, we recognize and differentiate between the proof-of-concept performance and clinically translatable performance in instances where comparisons exist, and where claims are inferred.

2 | EXOSOME HETEROGENEITY, CARGO-BASED CLASSIFICATION AND BIOMARKER SIGNIFICANCE

The biology of exosomes is highly heterogeneous and contextual to define, and vesicle capture and quantification nanochips are designed based on this variability.[16] Practically, many studies use the label exosomes specifically to denote small extracellular vehicles (EVs) that are enriched in canonical surface markers (CD9, CD63, CD81) and at the same time appreciate the fact that those vesicles overlap with other types of EVs as well as non-vesicular nanoparticles.[17,18] This heterogeneity has both opportunities and challenges as far as organization in diagnostic terms is concerned. It allows the disease states to be represented by changes in surface proteins, intraluminal proteins, microRNAs, messenger RNAs, lipids and in some instances, DNA fragments transported by EV fractions.[19] On the other hand, a single-marker capture methodology puts a threat of bias against certain subpopulation and a single-analyte result is susceptible to biological disparity. Multiplex architectures testing exosome identity markers and disease-associated markers at the same time favour single-drop devices, which is why it is a preferred alternative over high-complexity biomarker detection methods. Identity markers enable normalization and keep the sanity, disease markers help to classify and stratify risks.[20] These layers will increase the resistance to the vagaries of vesicle abundance as well as partially overcome under-representation of any particular subpopulation. Blood matrices of the whole-blood and plasma have highly abundant confounders capable of imitating and distorting actual signals of exosomes.[20]

Lipoproteins have similar size and density properties as small EVs and platelet-derived vesicles can become both more numerous in response to collection stress or inflammatory stimuli, and background proteins and microRNAs can be introduced by hemolysis.[21] These confounding entities might dominate in the limitations of a single drop format since large scale purification is not possible due to the sample volume available. Hence, an effective biomarker panel to use in order to study single-drop

exosomes should include disease-related biomarkers, as well as the ones that reflect contaminations and processing artifacts.[22] The predominant ones are lipoprotein-related markers, platelet-activation markers and haemolysis indicators and all of them serve as internal controls to put the multiplex readout in perspective.[23] Monitoring strategies prefer those markers that have measurably varying daily-to-weekly variations and correlate with therapeutic effect, whereas screening strategies prefer the use of very stable markers that discrimination between disease and non-disease phenotypes with high specificity.[23] Finally, the analytical work flow must not ignore the specific biochemical characteristics of the target analytes; proteins and RNAs vary in terms of stability, resistance to inhibitors added by anticoagulants, prelysis and precleanup requirements.

In turn, the choice of the platform must be in line with the desired level of biomarker: protein-centric panels can be used with affinity capture and immunoassays, whereas RNA-centric can be used with quick lysis strategies and inhibitor-tolerant amplification chemistries.[24] Even at microlitre level operation, any minor differences in surface adsorption, or the moment at which a valve acts or a channel wets can have significant consequences on yields therefore designs that reduce the number of interfaces and stabilize wetting behaviour are desirable. More importantly, realistic matrix conditions should be employed to substantiate claims of performance and not optimized laboratory buffers, because the effect of plasma proteins, lipoproteins and viscosity, both mitigate transport phenomenon and binding kinetics. In order to develop translational improvements, it is prudent to consider that analytical sensitivity and clinical utility should be separated; a moderately sensitive test can be useful of it is reproducibly responsive to clinical changes in the long run.[25] Where feasible, internal standards that cut across the workflow should be embedded within the platform in order to measure recovery efficiency and inhibition with respect to a per-run basis. The complex heterogeneity of extracellular vesicle biology, the challenges and opportunities for diagnostic applications, and the engineering design features are presented in Figure 2. This figure summarizes experimentally reported EV heterogeneity and overlapping characteristics of the different types of EVs, such as exosomes, small ectosomes, lipoproteins, protein aggregates and platelet-derived vesicles.[10–13] Normalization and enrichment signals include canonical EV identity markers (CD9, CD63 and CD81); disease-associated proteins, RNAs, lipids and other cargo classes are candidates that can be used for diagnosis and/or monitoring of disease.[11–14] In addition, blood-matrix confounders are defined as those that may alter exosome readout when measured in single drop assays and should therefore be monitored and included as internal standards, such as lipoproteins, haemolysis associated background and platelet activation.[15–17,23,24]

Because finger-prick sampling introduces user-dependent variability, robust cartridge designs should include passive metering, clot traps and controlled mixing zones to stabilize sample handling.[26] In an engineering sense the most common failure modes do not lack subcellular biomolecular interactions but mechanical and fluid-mechanical failures such as bubble formation, clogging, incomplete priming and intermittent flow apportionment.[27] Furthermore, manufacturing choices regarding the choice of thermoplastic material, surface activation chemistry and reagent storage conditions may change performance at the baseline; thus preliminary prototypes would need to expect scalability of both the material and process.[28] As a matter of fact, empirical, studies quantifying the proportion of relevant vesicles retained as well as the fraction of the contaminating particles separated together can offer most informative observation, with each of the parameters deciding on clinical applicability.[4,29] Deviations at the microlitre-scale operation, even in the minor deviations in the adsorption of the surfaces, the timing of the valve, or the wetting of the channels can result in observable changes in yields, creating a comparative advantage to the designs that reduce interfaces and stabilize wetting behaviour.[30] Combined, study built-in designs with clear and transparent controls and standardized metrology are plausible to performance verifications.

2.1 | Classification and biological significance of exosome subtypes based on cargo composition

Exosome heterogeneity is not only viewed in terms of vesicles and their size, density, cellular origins and surface-marker expression, but also in relation to the predominant molecular cargo loaded in the subpopulations of vesicles.[31] A key aspect of cargo-based classification is the fact that the biological role, diagnostic significance, isolation method and "sensing" chemistry are intrinsically tied to the location of a target signal within an exosome, whether it be on the membrane, within the lumen or within associated lipid and metabolites. Generally, different sorts of exosomes can be designated as protein-enriched, RNA-enriched or lipid/metabolite-enriched, DNA-containing or multi-omics exosomes.[31] Alternative classes of biomolecules can also be present in any particular vesicles, but these classification categories are useful for making choices of capture markers, vesicles lysis conditions, detection chemistries and normalization controls.

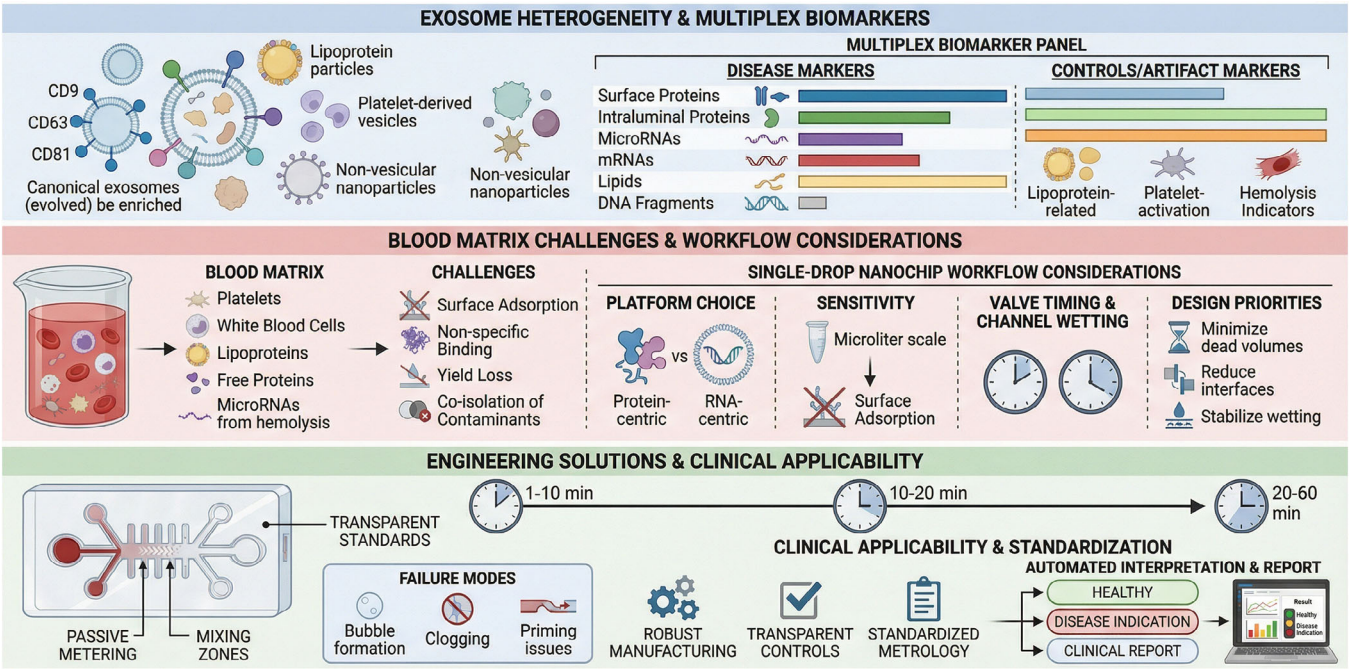


FIGURE 2 Evidence-linked map of extracellular-vesicle heterogeneity, biomarker classes and blood-matrix confounders.

Exosomes displaying protein-enriched structures include membrane and intraluminal proteins like tetraspanins (CD9, CD63, CD81), integrins, MHC molecular proteins, heat-shock proteins, enzymes, adhesion molecules, immune-regulatory proteins and disease associated antigens.[32] These proteins affect the uptake of vesicles, recognition of target cells, modulation of immune responses, antigen presentation, remodelling of the extracellular matrix, angiogenesis and metastatic organotropism. Due to the ability to access surface proteins without complete disruption of the vesicles, protein-enriched exosomes are particularly well suited for affinity capture and detection by immunoassays for diagnostic Nano chip applications. However, protein-based classification is inherently marker specific and could amplify (in exosome studies) the contribution of vesicles expressing the selected epitope, and hence over represent it, while at the same time underrepresenting marker-negative subpopulations of exosomes.[32]

Exosomes contain RNAs, including microRNAs, messenger RNAs, long non-coding RNAs, circular RNAs and other regulatory RNA species, that are enriched with RNA. Such RNA cargo can transform recipient cells and be responsible for tumour progression, EMT, immune evasion, inflammation, fibrosis, therapeutic resistance and tissue repair.[33] So exosome-based small RNAs can be of interest for monitoring diseases and molecular stratification, as long as proteins are not abundant, or where there is a change first in the gene regulatory mechanism, and only later in the phenotype. But efficient vesicle-lyse protocols are needed for nano chip workflows based on RNA, along with RNases inhibitors to prevent degradation and haemolysis-induced RNA backgrounds, and amplification chemistries that are compatible with anticoagulants and plasma inhibitors.[33]

Lipid and metabolite-enriched exosomes carry bioactive lipids, cholesterol, sphingomyelin, ceramides, phosphatidylserine, prostaglandin-related molecules, amino acids and metabolites that are able to change skin cell signalling, coagulation, inflammation, oxidative stress and metabolism in recipient cells.[34] Although these vesicles are of relevance in inflammatory diseases, cardiovascular disorders, cancer metabolism, tissue injury etc., measurement of them is more difficult in single drop devices as the lipoproteins also fall in the range of size and density as small EVs.[34] However, DNA carrying exosomes, such as those containing genomic DNA fragments, mitochondrial DNA or oncogenic DNA sequences, can offer extra insights into tumour burden, damage to the tissue and genomic instability; furthermore they should be carefully validated for their abundance and vesicular location.[35]

Cargo-based classification of exosomes is relevant from an engineering standpoint for single-drop microfluidic nanochips.[36] Protein-enriched exosomes are optimal for the following detection methods: immunoaffinity capture, aptamer-based detection, fluorescence or electrochemical immunoassays and multiplex surface-marker panels. The exosomes enriched with RNA need to be rapidly lysed, stabilized to preserve the RNA, subject to inhibitor-tolerant RT-qPCR, LAMP, digital PCR or CRISPR assisted

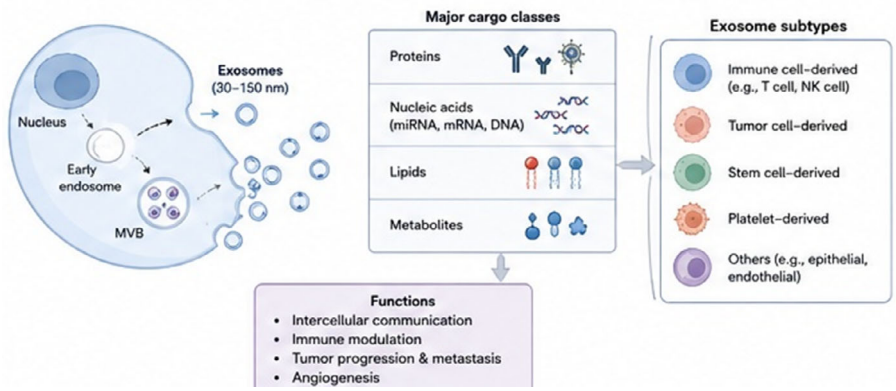


FIGURE 3 Exosome biogenesis, cargo-based classification and functional subtypes.

read-out. Ideally, the exosomes should be enriched for lipids/metabolites and there is need for orthogonal separation from lipoproteins, which could use markers previously identified by mass-spectrometry or label-free optical methods. Exosomes carrying DNA should be subjected to assays of nuclease-protection and assays to differentiate vesicle associated DNA from cell free DNA. Therefore, a clinically credible exosome nanochip should not have all exosomes as a uniform pool of biomarkers, but rather should express the type of desired cargo, capture logic, detection chemistry and contaminant-controlling strategy. Figure 3 summarizes the biogenesis of exosomes from endosomal compartments and highlights major cargo classes, including proteins, nucleic acids, lipids and metabolites. It also links cargo composition and cellular origin to biologically relevant exosome subtypes and functions, providing a framework for interpreting exosome heterogeneity in liquid-biopsy nanochip design.

Table 1 summarizes cargo-based exosome subtypes, highlighting their representative molecular contents, biological functions, diagnostic relevance and implications for designing microfluidic nanochips for multiplex liquid-biopsy applications.

3 | DESIGN PRINCIPLES OF MICROFLUIDIC NANOCHIPS FOR SINGLE-DROP OPERATION

To design a microfluidic nanochip to work on a single-drop basis, microfluidics, surface chemistry and sensing have to be treated as a single entity. The main goal is to move a small and variable biological sample through sequential unit operations while minimizing loss, contamination and assay variability.[37] Dead volume is a first-order effect at volumes of 10–50 μL: channels, connectors and capture chambers have to be designed to ensure the effective processing volume is equal to or close to the loaded volume, meaning that priming should only take a negligible portion of the sample. Passive flow methods such as capillary and pressure-based flow due to simple actuators may help simplify the system, but it should also be stable when haematocrit and viscosity change.[37,38] Getting to the capture surface is another bottleneck; in laminar microflows, the sheet down to the walls and the sheet outcroppings can slow down the binding of low-contraceptiveness targets, especially nanoscale.[38] These effects can be counteracted by the nano-bio interface engineering to supply sufficient surface area and increase collision frequency via nanowires, nanopillars, porous substrates, or patterned structures which cause local perturbations and reduce diffusion distances. Due to the presence of large proportions of protein in plasma, antifouling is mandatory and important nano-bio interface approaches that fasten capture and slow down fouling are demonstrated in Figure 4. Studies with 3D-nanopatterned microfluidic exosome capture[7] and studies with sensor-chip and polymer/zwitterionic coating and surface-control[28,29] provide supporting concepts for the nanostructured capture concept and the antifouling and surface-control strategies. The figure also emphasizes the importance of reporting capture efficiency, non-specific adsorption, vesicle integrity and co-isolated contaminants, as recommended in EV characterization and reporting recommendations.[23]

Some of the common antifouling approaches are polymer brushes, zwitterionic coatings, optimized blocking agents and regulated shear profiles, which reduce nonspecific adsorption, but not loquet of weakly injected target vesicles.[39] Initial considerations should be on release and downstream compatibility. When the chip uses strong affinity capture, there must still be an ability to access the cargo without necessarily employing extreme measures like analyte destruction, or sensor contamination, cleavable linkers, competitive elution, or gentle lysis-on-surface can be used to maintain the signal whilst allowing multiplex read-out.[40]

TABLE 1 Cargo-based exosome subtypes: Biological functions, diagnostic relevance and nanochip implications.

Cargo-based exosome subtype	Representative cargo	Main biological significance	Diagnostic relevance	Nanochip implication
Protein-enriched exosomes	CD9, CD63, CD81, integrins, MHC proteins, enzymes, tumour antigens, HER2, MUC1	Cell targeting, immune modulation, adhesion, angiogenesis, metastasis, antigen presentation	Useful for immunophenotyping, cancer subtype detection, monitoring treatment response	Best suited to immunoaffinity, aptamer capture, fluorescence/ electrochemical protein panels
RNA-enriched exosomes	miRNA, mRNA, lncRNA, circRNA	Gene regulation, EMT, immune evasion, inflammation, drug resistance, tissue repair	Useful for molecular stratification and longitudinal disease monitoring	Requires lysis, RNA stabilization, RT-qPCR/ddPCR/LAMP/ CRISPR-compatible workflow
Lipid/metabolite-enriched exosomes	Ceramides, cholesterol, phosphatidylserine, prostaglandin-related molecules, metabolites	Membrane signalling, coagulation, inflammation, metabolic reprogramming	Useful in inflammatory, cardiovascular, metabolic and cancer contexts	Requires strong lipoprotein controls because lipid-rich particles overlap with EVs
DNA-bearing exosomes	Genomic DNA fragments, mitochondrial DNA, oncogenic DNA sequences	Genomic instability, tissue injury, tumour-derived signalling	Potential complement to cfDNA liquid biopsy	Requires nuclease-protection controls and distinction from free circulating DNA
Multi-omic exosomes	Combined protein, RNA, lipid, DNA and metabolite cargo	Integrated disease-state signalling and recipient-cell reprogramming	Strongest for multiplex liquid biopsy and AI-assisted classification	Requires combined identity markers, disease markers and contamination markers

Lastly, design compatibility is predetermined by manufacturability and user workflow in overcoming lab boundaries. Cartridge systems have to be able to survive storage and shipping, have reagents in stable forms, and reduce the number of user steps.[41] The user interface should be simple and should have automated time and quality-control flags so that no report of a failed run is provided. Surface adsorption, timing of the valves or wetting of channels may vary mutually at the microlitre scale by small effects; designs to counteract these effects include designs which minimize surfaces and those that stabilize the wetting condition.[42]

Notably, performance claims are supposed to be made when the conditions are realistic in the matrices, as opposed to optimized buffers since the kinetics of movement and binding of plasma proteins, lipoproteins and viscosity could vary.[43] In the context of translation, an analytical sensitivity and clinical utility need to be differentiated as a medium-sensitive test can still be a good one provided it is reliable in its changes to be tracked to measure trends. In suitable cases, the platforms must include internal standards that cut across the workflow and result in estimation of recovery as well as inhibition of every single run.[44] Since finger-prick sampling results in a sources of variability depending on the operators, powerful cartridges will often have the capability to absorb timing jitter like passive metering, the existence of clot traps, or active mixing zones.[45] Regarding the mechanics and fluid Hamel forces engineering of the most prevalent failure modes are mechanical and fluidic as opposed to subtle biomolecular effects and includes bubbles, clogs, incomplete priming, and inconsistent partitioning of flows. Lastly, manufacturing decisions, such as which thermoplastic is used, activated surface, and reagent storage, could alter a baseline performance thus, initial prototypes should expect scalable materials and processes.[46]

At microlitre volumes, a slight variation in the adsorption of surfaces, timing of the valve or within the channel wetting can cause significant changes in the yields; designs that reduce interfaces and stabilize wetting are favourable.[47] Notably, realistic matrix conditions should be used to report performance claims as opposed to optimized buffers with plasma proteins, lipoproteins and viscosity having the potential to influence both the transport and binding kinetics.[48] In the case of translation, it helps to distinguish between analytical sensitivity and clinical utility where a test with an adequately sensitive level can still be of use in terms of monitoring patterns. Internal standards should be introduced where available into platforms

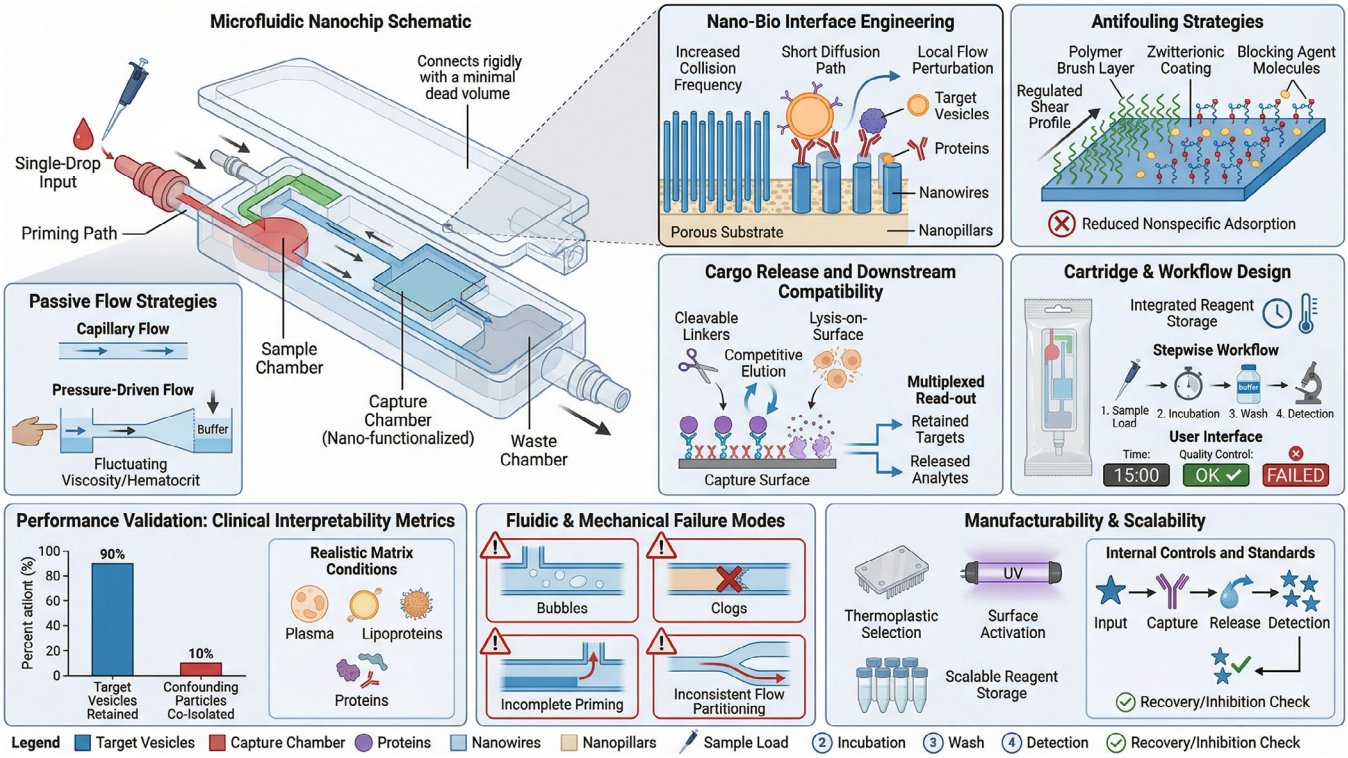


FIGURE 4 Evidence-linked nano-bio interface engineering for exosome capture. Schematic comparison of nanowires, nanopillars, porous surfaces and antifouling coatings used to increase vesicle–surface collision frequency, reduce boundary-layer limitations, and suppress nonspecific adsorption.

that cut across the entire workflow to provide estimation of recovery and inhibition per run.[48] In general, translation requires quality built-in designs that have clear controls and standard processes.

4 | EXOSOME ISOLATION STRATEGIES ON-CHIP: MECHANISMS, PERFORMANCE AND TRADE-OFFS

These mechanisms of on-chip exosome enrichment can be divided based on dominant physical or biochemical principles, with each providing a trade-off space that is characterized by purity, recovery, throughput and robustness.[49] The choice of a mechanism must start with application and matrix limitations being considered but not by individual performance measure.[49] The vesicles to be targeted by immunoaffinity capture include vesicles presenting particular surface epitopes and is addressed using antibodies, aptamers, and other immobilized ligands.[50] Such a methodology can be made highly specific and thus a richer clinically relevant subpopulation, which is of a great interest to an oncology or therapeutic response panel. However, it is also inherently marker-dependent and might miss vesicles without the selected markers, it also raises prices and makes it more susceptible to fouling especially with minimally processed plasma.[4,50] Membranes, nano-sieves, deterministic lateral displacement and other methods involving filtration are all sizes based strategies.[4] They are label-free and conceptually simple but in single-drop forms they may get clogged upon the exposure to whole blood or clumped proteins. In addition, lipoproteins have the same size regime, which restricts their purity without the use of orthogonal steps.[51] Figure 5 demonstrates the isolation trade-space, with a summary comparison being provided in Table 2 to aid the mechanism choice. Table 1 provides a benchmarking-oriented comparison of the major on-chip exosome isolation strategies. Because the available studies were not performed under identical conditions, the table should not be interpreted as a quantitative head-to-head ranking. Instead, it summarizes the practical trade-offs that should guide platform choice, including biological specificity, recovery, purity, analytical sensitivity, throughput, whole-blood compatibility, clogging risk, downstream assay compatibility, and the main evidence gaps that must be addressed before clinical translation.

Electrokinetic and dielectrophoretic methods make use of electric fields to isolate, sort or concentrate nanoparticles by charge, polarizability or induced motion across gradients.[58] These techniques are adjustable and can be combined with electronic detectors, but their effectiveness can be known to change as ionic strength, anticoagulant

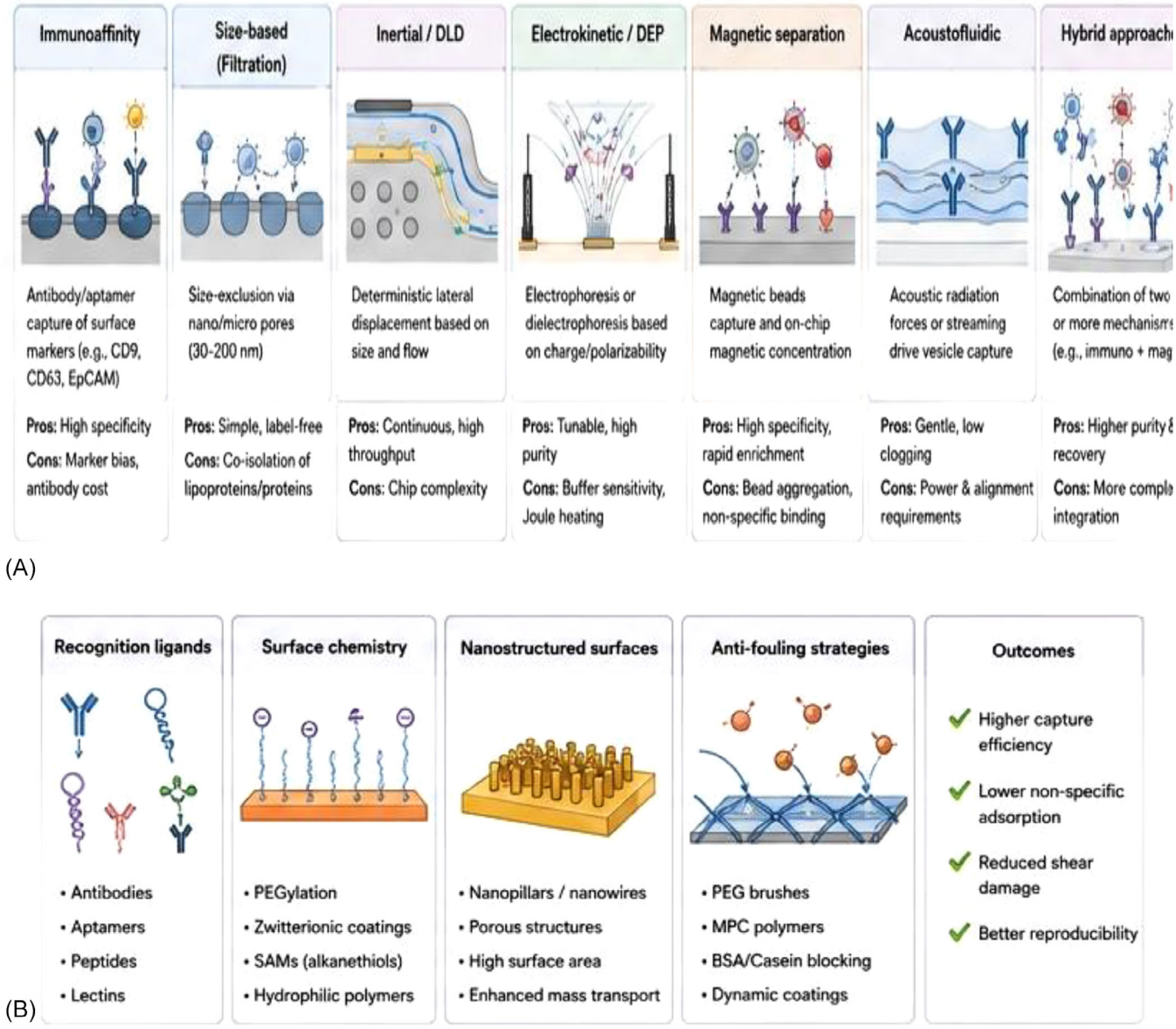


FIGURE 5 (A) On-chip exosome enrichment strategies and mechanistic trade-offs. DLD, deterministic lateral displacement; DEP, dielectrophoresis. The advantages and limitations shown are qualitative summaries intended to guide method selection for single-drop exosome nanochips. Actual performance depends on sample matrix, input volume, vesicle subtype, surface chemistry, flow conditions, downstream assay, and validation under whole-blood conditions. (B) Nano-biointerface engineering for efficient, selective and low-fouling exosome capture: the figure summarizes qualitative design principles rather than direct quantitative ranking. Capture performance depends on surface chemistry, ligand density, flow rate, shear stress, plasma-protein fouling, vesicle subtype, and downstream assay compatibility. Antifouling coatings and internal controls are required when translating these interfaces to whole-blood or minimally processed plasma samples.

chemistry and Joule heating, which are only differentially present in real samples. In acoustofluidic manipulation, acoustic radiation forces and streaming are used to put the particles apart in a gentle manner and this alternative often offers a lower risk of clogging compared to membrane-based approaches.[59,60] It can be perfectly compatible with whole-blood manipulation and can possibly maintain the integrity of vesicles but it might need more advanced hardware alignment and power control. Hybrid systems Hybrid systems employ orthogonal, for example, inertial plasma handling and subsequent affinity capture, or size pre-enrichment and subsequent electro kinetic concentration. The hybrid approach may be the most realistic option to progress into clinically useful, single-drop analysis of exosomes as it facilitates integration of orthogonal enrichment principles and can address challenges of any one of

TABLE 2 On-chip exosome isolation mechanisms: Strengths, limits and best-fit single-drop use cases.

Mechanism	Representative quantitative performance metrics	Recovery efficiency / yield	Purity / selectivity	Limit of detection / analytical sensitivity	Throughput / sample volume / time	Whole-blood compatibility	Lipoprotein-control strategy	Main evidence gap	Translational readiness	References
Immunoaffinity	3D nanopatterned immunocapture chip detected tumour-associated exosomes at 10 exosomes μL^{-1}, approximately 200 vesicles in 20 μL spiked plasma/sample.	NR or marker-dependent; generally medium–high when target epitopes are abundant	High for selected antigen-positive vesicle subtypes; lower for unbiased EV profiling	10 exosomes μL^{-1} in a representative 3D-nanopatterned platform	Few-microlitre to tens-of-microlitre plasma inputs; assay time varies by capture/sensing design	Medium	Use CD9/CD63/CD81 plus disease-marker co-capture; include ApoA1/ApoB channels as exclusion or correction markers.	Limited subtype coverage; CD9/CD63/CD81 or disease-marker bias; recovery often not reported under whole-blood conditions	Medium–High	[9,52]
Size-based membranes / nano-sieves	ExoDisc-type size-selective platforms report EV isolation in ≤30 min, with reported recovery up to ~95%; Exo-CMDS enriched exosomes from < 300 μL blood in 8 min, with an optimal concentration of 5.1×10^9 particles mL^{-1}.	Up to ~95% in representative centrifugal nanofilter systems	Low–Medium; size overlap with lipoproteins, protein aggregates, and non-exosomal EVs limits purity	NR for isolation alone; downstream aptamer fluorescence detection reported sensitivity improvement versus ELISA	8–30 min; <300 μL blood or raw biofluid inputs reported	Low–Medium	State clearly that size alone is insufficient; recommend SEC, affinity capture, or depletion as an orthogonal step.	Poor discrimination from lipoproteins/protein aggregates; clogging and pressure instability in minimally processed blood	Medium	[53]
DLD / inertial fractionation	Thermally oxidized tapered DLD arrays achieved > 90% purity in nanoparticle and exosome separation experiments.	NR or medium; depends strongly on array geometry, flow rate, and pre-processing	>90% purity reported in representative DLD exosome experiments	NR	Generally high-flow potential, but single-drop performance depends on pressure stability and fabrication precision	Medium–High	Use as upstream pre-enrichment only; validate output with ApoA1/ApoB and albumin measurements.	Requires precise fabrication and sample-flow control; limited standardized data in finger-prick whole blood	Medium	[54]
Electrokinetic / DEP	ExoDEP-chip reported an analytical detection limit of 193 exosomes mL^{-1} using microsphere-mediated DEP isolation with immunoaffinity fluorescence detection.	NR or medium–high under controlled conductivity	Medium; selectivity depends on dielectric contrast plus downstream affinity detection	193 exosomes mL^{-1} in representative ExoDEP-chip format	Rapid microfluidic concentration/detection; exact throughput varies by field strength and chip design	Low–Medium	Combine physical concentration with downstream EV-marker confirmation; test conductivity effects in lipemic plasma.	Sensitive to ionic strength, Joule heating, anticoagulants, electrode fouling, and sample conductivity	Low–Medium	[55]

(Continues)

TABLE 2 (Continued)

Mechanism	Representative quantitative performance metrics	Recovery efficiency / yield	Purity / selectivity	Limit of detection / analytical sensitivity	Throughput / sample volume / time	Whole-blood compatibility	Lipoprotein-control strategy	Main evidence gap	Translational readiness	References
Acoustofluidics	Integrated acoustofluidic whole-blood platform reported > 99% yield for 110-nm particle isolation, 98.4% exosome purity, and > 99.999% blood-cell removal; FLOAT-type acoustic trapping reported > 90% EV yield in < 10 min.	>90% to > 99% in representative acoustic EV/nanoparticle systems	Up to 98.4% exosome purity reported in acoustic exosome isolation module	NR for biomarker detection; mainly isola-tion/enrichment metrics reported	<10 min in acoustic trapping formats; continuous-flow potential	Medium–High	Emphasize low-clogging whole-blood handling but require lipoprotein-marker validation of enriched fractions.	Hardware alignment, acoustic power control, heating, and reader complexity; fewer standardized clinical matrix comparisons	Medium	[56]
Hybrid platforms	Hybrid systems combine orthogonal enrichment and detection; representative examples include size/enrichment modules plus affinity or optical/electrochemical sensing. Quantitative performance is highly platform-specific.	Potentially high, but must be reported per module and end-to-end	Potentially high when orthogonal size, affinity, and contamination controls are combined	Depends on integrated sensor; can range from vesicle-counting sensitivity to biomarker-specific LoD	Variable; integration may reduce transfers but increase assay time and failure points	Potentially high	Present as the strongest approach when they combine cell/plasma handling, lipoprotein depletion or fractionation, EV-marker capture, and contamination-aware readout.	Few direct standardized head-to-head comparisons using the same whole-blood matrix, input volume, EV standard, recovery definition, purity definition, and readout	Medium–High, but evidence-dependent	[57]

Note: Quantitative values are representative examples from cited or closely related microfluidic EV studies and should not be interpreted as direct head-to-head comparisons, because studies differ in sample matrix, input volume, EV standard, recovery calculation, purity definition and downstream readout.
Abbreviations: DLD: deterministic lateral displacement; DEP: dielectrophoresis.

the approaches. However, very few studies directly compare hybrid, immunoaffinity, size based, electrokinetic and acoustofluidic platforms with the same sample type (cells), same input volume, vesicle standard and performance metrics. So, it is accepted as an engineering expectation that hybrid systems will be superior, and not something that we have been proven generally. Future investigations should compare the techniques head-to-head in realistic whole blood or finger prick plasma environment.[61,62]

The potential changes in surface adsorption, valve timing or channel wetting are accented at the level of the microlitre; therefore, designs with reduced interfaces and one that tries to stabilize wetting are beneficial.[63] Notably, the performance claim must be carried out under realistic matrix conditions instead of in optimized buffers since the plasma proteins, lipoproteins and viscosity are known to impact both the transport and binding kinetics. In the case of translation, analytical sensitivity should not be equated with clinical utility, because, a modestly sensitive assay can still be of benefit, provided that it can faithfully indicate the trends in patient monitoring. The internal standards need to be built into the platforms where feasible, and should crosscut the entire workflow, in order to estimate recovery and inhibition at each run. Due to the fact that finger-prick sampling brings about variability by users, robust cartridges tend to add mechanisms to correct variability in timing, for example, passive metering or clot traps or controlled mixing zones.[64] The most frequent failure modes are not associated with subtle biomolecular effects but mechanical and fluidic in nature (which include bubbles, clogs, incomplete priming and asymmetric flow partitioning).[65]

Lastly, the manufacturing decisions including thermoplastic selection, surface activation and storage of reagents can alter baseline performance hence early prototyping needs to predict scalable material and processes.[3] At microlitre scale, even small changes in surface adsorption, valve timing or channel wetting can induce significant changes in yields and so designs that reduce interfaces and stabilize wetting are therefore beneficial.[66] Notably, performance claims also must be reported at real conditions at the matrix level as opposed to using optimized buffers because plasma proteins, lipoproteins and viscosity have the potential to change transport and binding kinetics.[66] For translation, analytical sensitivity should be distinguished from clinical utility; a moderately sensitive assay may still be valuable if it reliably tracks clinically meaningful trends. Comprehensive vigorous combination designs that have clear controls and standard metrology permits plausible single-drop exosome testing to be utilized in the peripheral clinical determination of various populations of patients. Table 1 complements Figure 5A compares major microfluidic exosome-enrichment strategies, including immunoaffinity, size-based filtration, inertial/DLD separation, electrokinetic/DEP enrichment, magnetic separation, acoustofluidics and hybrid approaches. It highlights their working mechanisms, strengths, limitations and practical suitability for single-drop liquid-biopsy nanochips. Representative supporting studies include nanostructured immunoaffinity capture,[7,39,50] size-based and membrane/nano-sieve isolation,[24,49,61] microfluidic particle separation and inertial/DLD principles,[44,62] electrokinetic and DEP approaches,[41,43,58] acoustofluidic manipulation,[42] and integrated or hybrid microfluidic systems.[3,5,8] Because most studies differ in matrix, input volume, vesicle standard and purity definition, the figure should be interpreted as a mechanism-level comparison, not a quantitative meta-analysis.

4.1 | Critical comparison of on-chip isolation strategies

Several on-chip protocols have been suggested for enrichment of exosomes, but it is difficult to compare directly the performance of these protocols since each of them employs different sample (cell, tissue, blood, urine, etc.), different cell number, vesicle standard (density, diameter, etc.), recovery estimation, purity definition and readout. Immunoaffinity approaches are normally the most powerful for achieving a level of biological specificity, typically involving the enrichment of vesicles with selected markers the case studied the extraction of vesicles enriched in either CD9, CD63, CD81, or disease-associated epitopes.[67] This specificity, however, brings in the bias on the markers and could lead to under-representation of the vesicle subpopulations that do not have the chosen surface proteins. Immunoaffinity systems are thus most appropriate if the intended clinical question is related to a specific vesicle subtype whereas unbiased EV profiling is much less suited to immunoaffinity.

Label-free methods like size-based approaches, such as membranes, deterministic lateral displacement, and inertial fractionation are beneficial and can be coupled to sensing modules upstream. They have one major drawback—size does not differentiate between the exosomes and other extracellular particles with similar size dimensions, such as lipoproteins, protein aggregates, etc.[68] These methods may also lead to clogging and/or loss of purity along with pressure instability in minimally processed plasma or whole blood. Therefore, size-based methods are suitable for pre-enrichment and are most often followed by orthogonal purification or identification for clinically interpretable measurement of the biomarker.

Programmable concentration and compatibility with electronic sensors are feasible using two methods:

electrokinetic and dielectrophoretic, which are, however, highly sensitive to ionic strength and conductivity, joule heating, and anticoagulant chemistry and electrode fouling.[69] Such dependencies make them strong tools in controlled matrices but the inclusion of a robust sample conditioning and thermal control make them more of a challenge in variable finger-prick blood samples. For handling whole blood, comparatively gentle and less clogging acoustofluidic techniques are attractive but may necessitate more complicated equipment, acoustic focussing and the control of acoustic power compared to passive microfluidic techniques.[70]

It is therefore not correct to regard hybrid systems as necessarily better but as rather an engineering solution to the limitations of the separate systems. For instance, reducing the cellular and protein load upstream of immunoaffinity capture by a size and/or inertial separation step, and quantifying the cellular and/or protein load downstream using electrochemical and/or optical readout. Orthogonal selective mechanisms can be used together on hybrid platforms, but the drawback is that devices are complicated, it is a burden to manufacture, the assay time is extended and there is a risk of either losing samples or having them lost over time. Claims that hybrid systems are most clinically credible should be presented as a hypothesis that is supported by engineering logic, while not a proven conclusion, unless direct head-to-head comparisons are available for systems under identical conditions (with the same blood-matrix).

Experiments have been conducted showing that hybrid or integrated systems are practical and worthwhile, if not universally valuable over all single-mechanism systems. For instance, the ExoSearch chip achieved the step of the combined enrichment by immunomagnetism with in situ multiplexed detection of plasma exosomes and showed ovarian-cancer associated exosome-marker profiling in the plasma samples with very small volume.[71] Integrated magneto-electrochemical platforms (like iMEX/HiMEX-type assays) also demonstrate that the combination of magnetic enrichment of EVs and electrochemical detection could shorten the workflow and enhance the clinical feasibility; one such assay was able to profile tumour proteins bound to EVs from plasma in less than an hour and achieve highly accurate diagnosis of colorectal cancer samples.[72] More recently, EV-Blade integrated the whole-blood centrifugation, plasma filtering, size selection, exosomes capture by immunomagnetic selection, and on-chip lysis of the exosome in an automated cartridge, achieving exosome purity 'similar' to the manual size and affinity platform and with less lipoprotein contamination than SEC-only methods.[4,73] Such studies are one way of supporting the idea that hybrid systems offer the possibility of both lowering transfer losses, and facilitating the ability to merge orthogonal enrichment principles with detection/molecular analysis.

Because published microfluidic exosome studies differ in sample matrix, input volume, vesicle source, recovery calculation, purity definition and downstream readout, direct numerical ranking of isolation technologies is currently not possible. A clinically useful comparison therefore requires a standardized benchmark framework. At minimum, immunoaffinity, size-based, electrokinetic/dielectrophoretic, acoustofluidic and hybrid platforms should be compared using the same input matrix, preferably fresh anticoagulated whole blood and matched plasma, the same input volume range relevant to single-drop testing, the same vesicle reference material or clinical sample set, and the same definitions of recovery, purity, throughput, limit of detection, clogging rate and downstream assay compatibility.[73] Under such a framework, immunoaffinity capture would be expected to perform best when the clinical question targets a defined antigen-positive vesicle subpopulation, but it may underrepresent marker-negative vesicles and is vulnerable to fouling. Size-based methods are attractive for label-free pre-enrichment and simple integration, but their ability to distinguish exosomes from lipoproteins, protein aggregates and similarly sized extracellular particles is limited. DEP and other electrokinetic methods offer tunable concentration and compatibility with electronic detection, but their performance depends strongly on conductivity, anticoagulant chemistry, electrode stability, and thermal control.[73] Acoustofluidic methods are promising for gentle and low-clogging enrichment from complex samples, but they require careful control of acoustic alignment, power, heating, and device-to-device reproducibility.[74] Hybrid platforms may provide the strongest clinical path when they combine orthogonal separation principles with internal contamination controls; however, they should not be assumed to be superior unless tested against single-mechanism devices under identical blood-matrix and workflow conditions. Thus, platform selection should be driven by intended use: immunoaffinity or hybrid systems for subtype-specific disease monitoring, size-based or inertial methods for upstream enrichment, electrokinetic methods for electronically integrated concentration and sensing, and acoustofluidics for applications prioritizing gentle handling and reduced clogging risk.

In terms of improvements in performance, the general trend on the reported microfluidic platforms has been to either increase the amount of vesicle-surface interaction, introduce additional orthogonal separation steps, and/or ensure greater tolerance for the matrix. In the early approaches, focussed on size-based and membrane/nanosieve systems, priority was given to rapid and label-free enrichment (up to approximately 95% recovery

and 8–30 min processing), though at the cost of relatively low level of purity due to co-isolation of lipoproteins and protein aggregates. Nanostructured immunoaffinity systems enhance analytical sensitivity due to an increase in capture surface area and vesicle collision frequency, but there is limited information on the recovery of the exosomes, depending on the marker, and on the robustness using whole blood.

DLD/inertial approaches achieve improved purity through particle separation based on the geometry of the system and demanded exact fabrication and flow control with a representative purity exceeding 90%. With the aid of field-assisted concentration, LOD was also improved with the use of electrokinetic/DEP systems to achieve a representative detection level of 193 exosomes mL^{-1}; however, these systems were still affected by conductivity, joule heating, the presence of anticoagulants and electrode fouling. Positive contributions to whole-blood compatibility were made by the acoustofluidic systems, which report isolation of particles as large as 110 nm with yield > 99%, purity of exosomes > 98.4%, blood-cell removal > 99.999%, and EV yield > 90% in < 10 min for representative setups. In general, the field has progressed from enrichment based on a single principle to hybrid enrichment cartridges that integrate plasma handling, pre-enrichment, affinity or physical concentration, detection of contamination in the sample and internal quality controls. These systems are attractive, but quantitative ranking have constraints due to the use of different matrices, different volumes of EV's used, different definition of purity, and different assays used downstream.

4.2 | Expanded Comparison of On-Chip Exosome Enrichment Strategies

Another key method of exosome enrichment is magnetic microfluidic separation.[75] This involves coating magnetic beads or nanoparticles with antibodies, aptamers, peptides, or other ligands that bind to EV markers like CD9, CD63, CD81, EpCAM, HER2, MUC1, PD-L1 or other disease markers. Once bound, an external magnetic field will then concentrate or immobilize the bead–exosome complexes for washing, the amplification of the signal, or lysis or even the direct detection of the beads.[75] The major benefits of magnetic separation include high biological specificity and compatibility of the process with small-volume samples, high enrichment-times, convenience in integrating with electrochemical/electro-optical detection, and the capacity to create multiplex panels due to the different types of beads which can be encoded.[76] It is particularly helpful if the clinical aim is specifically that of enriching a defined subset of exosomes that is antigen positive as opposed to the overall pool of EVs. But this specificity also introduces some constraints: This specificity can be problematic because vesicles that do not contain the marker of interest cannot be detected, beads can aggregate reducing the reproducibility of the results, beads can carry over to the optical or electrochemical readout, and there is the possibility of nonspecific adsorption that raises background signal in plasma-rich samples. As a result, magnetic enrichment should be used in conjunction with EV identity markers, contamination markers and recovery controls.

A second widely adopted strategy that must be contrasted with off-chip centrifugation is centrifugal microfluidic separation, either in the form of lab-on-disc or centrifugal-pneumatic cartridge. Built around the principle of controlled rotation, centrifugal microfluidics enables the separation, metering, valving, filtration, density/dimension based fractionation, reagent mixing, immunocapture, washing and lysis of plasma in a closed cartridge without the need of ultra-high speed ultra-centrifugation.[4] This format is very appealing to sample-to-answer testing of exosomes as multiple unit operations can be automated in a small-size device, limited hands-on and minimal contamination risk. Centrifugal systems are also suitable for upstream blood processing and downstream exosome enrichment and molecular detection. However, drawbacks include requiring special spinning readers, complexity of their fabrication and valving, sensitivity to disc balancing and rotational programming, potential loss of vesicles in interfaces, and necessitating their validation for shear, clogging and recovery under whole-blood conditions. Therefore, centrifugal microfluidics is better suited for situations where workflow integration and automation are most important rather than maximum molecular sensitivity is desired.

Overall, each enrichment strategy occupies a different trade-off space. Immunoaffinity and magnetic methods are very biologically specific but can introduce a bias toward marker-positive vesicles. Label-free and simple separation methods such as size-based and inertial method are prone to failure of distinction exosomes from lipoproteins and protein aggregates. Tunable concentration and electronic integration are offered by electrokinetic and dielectrophoretic systems, which, however, are subject to conductivity, joule heating, electrode fouling and anticoagulant chemistry. Acoustofluidic approaches offer gentle, low-clogging manipulation but require careful acoustic alignment and power control. Strong automation potential exists with centrifugal microfluidics, but only specific instruments are available, and the construction of cartridges is robust. The hybrid platform combines two or more mechanisms and would only be considered superior

under identical sample matrices, input volumes, recovery definitions, purity metrics and downstream readouts.

Figure 5B illustrates how engineered nano-biointerfaces improve exosome capture in microfluidic nanochips by increasing surface area, enhancing vesicle–surface collision frequency, shortening diffusion distances and reducing nonspecific adsorption. It compares nanowires, nanopillars, porous substrates, patterned capture surfaces, and antifouling coatings as practical strategies for improving capture efficiency and assay reproducibility

4.3 | Practical mitigation of lipoprotein interference in whole-blood exosome nanochips

Single drop exosome testing is one of the most difficult purity issues, as there is some overlap in the hydrodynamic diameter, buoyant density, refractive index, surface charge and lipid/protein composition between HDL, LDL, VLDL, the chylomicron remnants, protein aggregates and small EVs.[4,77] Thus, it cannot be assumed that only one physical characteristic, most importantly, size, would indicate an exosome-specific signal in plasma or whole blood. Realistic whole blood conditions will necessitate an orthogonal approach to lipoprotein mitigation that combines pre-analytical control, lipoprotein fractionation or depletion, selective capturing and signal interpretation in the presence of lipoproteins.

For some lipoprotein-rich fractions, density-gradient ultracentrifugation or iodixanol/sucrose gradient can separate some EV-enriched fractions, making density-based separation a good reference method.[4] Some small EVs are however possibly considered to overlap with HDL and the approach is hard to miniaturize in fast single drop cartridges.[78] Thus, density-gradient can be a more effective benchmarking/comparator method for microfluidic translation as a point-of-care module. For the single-drop platforms, it is important to compare recovered fractions to other density-gradient or orthogonal comparison methods so the amount of ApoA1-, ApoB-, and albumin-positive material remaining after chip processing can be calculated.

Another practical method is size-exclusion chromatography (SEC), which allows for the exclusion of the soluble protein background, and allows for the separation of the vesicle-enriched fractions from the fractions of proteins which can elute later.[4] However, SEC is not capable of a complete separation of EVs from similarly sized lipoproteins, particularly those with HDL size. The pore size, column geometry, flow rate, fraction window and sample dilution for microfluidic or miniaturized SEC approaches should therefore be optimized and the samples chosen after SEC and containing EVs should be verified by EV markers and lipoprotein markers. SEC is most useful for single drop work flows as upstream clean up or pre-enrichment prior to affinity capture or multiplex detection than as a purification technique by itself.

A more exosome-directed approach is to use dual-marker or multi-marker affinity selection. To exclude or flag ApoA1-, ApoB-, or albumin-enriched events, platforms can enrich vesicles expressing the canonical EV markers, CD9, CD63 or CD81, instead of particle selection based on size or for one tetraspanin alone.[4] This can be achieved by a combination of sequential and simultaneously acquired channels, sandwich detection mechanism, single particle / multiplex gating (IP-valid detection from an EV identity marker channel and a disease associated marker channel with lipoprotein associated channels acquired as exclusion / normalization markers). This design limits to a minimum the lipoprotein-derived false positives but could lead to bias toward marker-positive EV subpopulations and should thus be reported as such.

A high-resolution separation of nanoparticles can be enabled by asymmetric-flow field-flow fractionation and related field-flow fractionation (FFF).[79] These techniques are able to separate more effectively the EV subpopulations and lipoprotein-associated particles and need careful standardization of the methods and specialized instrumentation. Single-drop nanochips are immediately useful to serve as orthogonal validation and calibration methods, whereby chip-isolated fractions can be compared to AF4-resolved fractions to assess if apparent biomarker signals are from single EV-enriched fractions or lipoprotein type fractions.

The depletion can, additionally, be performed lipoprotein-specifically prior to the extraction of EVs. Downstream depletion of lipoproteins (HDL/ApoA1+, LDL/VLDL/ApoB+) can be accomplished using antibody or aptamer or beads prior to enrichment.[80] Magnetic or affinity depletion module can be applied in microfluidic cartridges which is attractive because these can be placed up the flow from EV capture zones. However, the method might compromise the recovery of EVs, or there may be loss of apolipoproteins that are located on or in EVs; hence, validation needs to be implemented with respect to EV recovery, and not just lipoprotein depletion. Thus, a practical design of such a cartridge could involve the first zone consisting of ApoA1/ApoB-rich particles capturing, the second zone of CD9/CD63/CD81 or disease marker capturing, and the third zone, multiplex-detecting EV markers, disease markers and left over lipoprotein markers.[80]

In the case of single-drop systems for translation, lipoprotein interference should be considered a failure-in-measure, not just a theoretical limitation. Studies should, at a minimum, report recovery of EVs along with ApoA1,

ApoB, and the normalization by albumin, total protein and particle-to-protein ratios, test lipemic, haemolysed and platelet rich samples, include negative controls with lipoproteins and report a rejection or correction rule for samples that have too much lipoprotein signal. However, clinically useful microfluidic systems will likely involve hybrid workflows, where it will be necessary to handle whole-blood plasma, purify or fractionate lipoproteins, capture EVs by means of the presence of exosome markers, detect markers associated with various diseases, and implement the algorithmic gating that will discriminate against signals derived from ApoA1/ApoB while allowing acceptable exosome recovery.

4.4 | Translational maturity and manufacturability potential of microfluidic exosome platforms

Whilst microgravity exosome devices have demonstrated good analytical capabilities, many substantial variations in the degree of maturity for translation and the ease of manufacturability exist.[81,82] To explain, there are three general levels of the technologies. Early stage proof-of-concept systems encompass highly specialized nanostructured immunocapture devices, electrokinetic/dielectrophoretic concentration chips, plasmonic or field-effect transistor-based sensors (FET-based sensors), and complex molecular barcode or CRISPR-assisted readout.[81,83] They usually achieve a high sensitivity under laboratory conditions, but their scalability is hampered by manufacturing difficulties, structural defects of manufactured surfaces, stability of electrodes or nanostructured surfaces, inclusion of reagents and lack of their validation in realistic whole-blood matrices. Second, intermediate stage platforms are the size based, inertial, deterministic lateral displacement, acoustofluidic, bead based, fluorescence-array and electrochemical-array platforms.[84] These technologies show improved life-lines to integration into cartridges but need to improve evidence for clogging control, lot to lot reproducibility, robustness in whole blood and scalable reader design. Third, cutting-edge low-complexity plasma-handling technologies are coupled with orthogonal EV enrichment, contamination advent quality control, stable dried reagents and small optical or electrochemical detection all in one integrated cartridge.[85] However, such systems are only considered to be amenable to translation when they show end-to-end performance using realistic whole-blood samples over multiple manufactured lots of chips, operators and test sites. Thus, clinical readiness should be considered in the context of the analytical sensitivity, presence of scalable materials, manufacturable cartridge architecture, stability of reagents, automated operation, quality-control thresholds and reproducible clinical performance at intended use conditions.

5 | ON-CHIP LYSIS AND MOLECULAR ACCESS: MAKING CARGO MEASURABLE

After enrichment, the apparatus has to supply a measurable cargo but leave out signal integrity and artefactual contributions. Even the losses associated with lysis and subsequent clean-up can be the same as the analyte budget in single-drop workflows; thus, the strategy used in lysis is necessarily subject to optimization with the sensing chemistry and that biomarker of interest. Chemical lysis is fast and easy to use with a set of readily available reagents but the addition of detergents and chaotropes can interfere with immunoassay performance, increase optical background and destroy the stability of electrochemical surfaces.[86] Less complexity in reagents is provided by thermal lysis, but temperatures higher than this may lead to protein denaturation and premature RNA degradation unless the temperature is carefully monitored.[87] Electrical lysis is rapid and localized, but it may introduce electrode artifacts, pH shifts, or reactive species that interfere with protein or nucleic-acid measurements.[87] Ultrasonic or mechanical disruption is also a viable option, but this has a risk of heating, fragmentation and integration of low cost cartridges. Single-drop nucleic-acid protocols often rely on chemistries that tolerate immobilizable inhibitors and minimize transfers, partitioned or digital methods improve quantitation and avoid the biases of sensitive matrices, isothermal amplification has less hardware and shorter assay times, and many more.[88] The list in Table 3 has been compiled with lysis and cargo-access options, and marks compatibility threats which often determine how performance is done in practice.

The main issues in the development of protein panels are nonspecific adsorption, cross-reactivity which grows with the complexity of the panel and the limited plasma matrix dynamic range. Strong designs thus combine surface chemistry of antifouling, intelligent approach to choice of antibody-pairs and calibration schemes that rectify memory effects and matrix effects. In both the biomarker modalities, internal controls cannot be done without.[99] Spike-in standards can be used with spike-in quantifying recovery and isotype controls can be used with estimating nonspecific binding, contamination markers can show if a sample is dominated by lipoproteins platelets or haemolysis-derived background. In the absence of such controls, multiplex signatures can be convincing in discovery cohorts but fail when trying to be used in clinical settings.[100] In practice, most informative studies both measure the fraction of the concentration of the target vesicles

TABLE 3 Lysis and cargo-access strategies: Compatibility and risks in integrated nanochip workflows.

Lysis/cargo access method	Advantages	Key risks	Best paired with	References
Chemical lysis (detergents/chaotropes)	Fast; low-power; reagent-stored	Detergent interference; background increase; disrupts surface epitopes	Optical arrays; bead assays with tolerant chemistry; downstream nucleic-acid amplification	[89]
Thermal lysis	Simple hardware; easy integration	Protein/RNA damage if overheated; bubble formation	Electrochemical readouts with controlled heating; thermostable enzymes	[90]
Electrical lysis (electroporation/electrolysis)	Localized; rapid; low reagent load	Electrode artifacts; oxidation; pH shifts	Electronic sensors with isolation steps; redox-buffered chemistries	[91]
Mechanical/sonic (acoustic/ultrasonic)	Effective disruption; rapid	Heating; fragmentation; device complexity; aerosol risk (off-chip)	Pre-validated RNA assays; robust chips with thermal management	[92]
Freeze–thaw cycling	Reagent-free; simple concept	Variable efficiency; vesicle aggregation; cargo shearing; slow	Off-chip prep + on-chip detection; protein-lean workflows	[93]
Osmotic shock (hypotonic)	Mild; preserves some protein function	Incomplete lysis; dilution effects; salt rebalancing needed	Enzyme-based assays; fluorescence readouts tolerant to ionic shifts	[94]
Enzymatic permeabilization (proteinase K, RNase-free proteases; mild surfactants)	Tunable; can enrich for protected cargo (inside vesicles)	Over-digestion; batch variability; enzyme carryover inhibits assays	Nucleic-acid workflows with cleanup; digital PCR/LAMP after inhibition control	[95]
Photothermal lysis (laser + plasmonic nanostructures)	Highly localized; fast; minimal bulk heating	Requires optics; nanomaterial variability; photo-bleaching	Optical detection platforms; plasmonic sensors already in chip	[96]
Nanopore/chemical pore-formers (e.g., saponin-like; membrane-active peptides)	Partial permeabilization; preserves large complexes	Non-specific leakage; pore-former interference; safety/handling	Protein assays where intactness matters; sequential surface→cargo workflows	[97]
Solid-phase capture then on-bead lysis	Concentrates EVs; reduces matrix effects; modular	Bead carryover; nonspecific adsorption; variable capture efficiency	Immunocapture + RT-qPCR/ddPCR; electrochemical bead-based sensing	[98]

retained and the fraction of confounding particles that do co-isolate as both are a measure of clinical relevance.[101] Although surface adsorption, valve timing, or channel wetting can be varied on a microlitre scale, to be measurably different, and thus highly sensitive designs that reduce interfaces and stabilize wetting hold a special benefit. Notably, realistic matrix conditions should be ideal in developing performance claims as opposed to optimized buffers since the viscosity, performance of lipoproteins and plasma proteins can change both transport and binding kinetics.[102]

When working in the field of translational applications it can be helpful to identify the difference between analytical sensitivity and clinical utility as a test with moderate sensitivity can be useful in providing trends in measurement. Internal standards should also be added between platforms where possible in order to estimate recovery and inhibition of each individual run.[103] Since finger-prick sampling induces variability with the use of the user, resilient cartridges may include technology to counteract kinetic differences, including passive metering or clot trap or controlled mixing region.[104] Mechanical and fluidic problems, such as bubbles, clogs, incomplete priming and uneven partitioning of the flow are the most prevalent ways of failure, not sensitive biomolecular perturbations, though they come in engineering perspective.[65] Lastly manufacturing choices regarding thermoplastic choice, surface activation and reagent storage may alter baseline performance thus initial prototypes ought to expect scaled materials and processes.[105] Experimentally the most informative studies give quantitative measurements of both the fraction of target vesicles retained and the fraction of

confounding particles co-isolating, which should be combined together to give clinical interpretability.

Microlitre scale between the microlitre and nanolitre scale, small changes in the surface adsorption, valve timing, or channel wetting can introduce significant changes to yields; designs that reduce amounts of interface and wetting have demonstrably significant advantages.[106] Reporting the performance claims under realistic matrix conditions in place of the optimized buffers is also required because the plasma proteins, lipoproteins, and viscosity may alter the transport and binding kinetics.[107] To be used translationally, it is still beneficial to uncouple analytical sensitivity and clinical utility, and appreciate that a modestly sensitive test may be of good use when it is reliably used to track trends. Where possible, internal standards should be embedded on the platforms, which will accompany the sample throughout the workflow to easily estimate recovery and inhibition on a run-by-run basis.[108] Altogether, credible single-drop exosome testing with either transparent controls or standardized metrology is supported by integrated designs, which integrate both transparent controls and standardized methodology to support decentralized clinical decision-making. Table 3 links lysis choice to assay interference risk and to the practical constraints of integrated systems.

6 | MULTIPLEX BIOMARKER PROFILING MODALITIES INTEGRATED ON NANOCHIPS

Multiplex sensing on nanochips can be developed in two main directions, architecture and transduction.[109] Architectures determine how many analytes can be measured at a given time without requiring prohibitive increases in physical size or complexity of the analytical processes, transduction properties determine what is necessary of a reader, its stability and foulability.[109] Spatial arrays assign each target to a distinct capture site and are easily interpretable, but the size of the footprint increases with the size of the plex, inter-spot manufacturing variability may be a source of systematic bias, and it needs to be strictly managed.[109] Spectral multiplexing, conversely, uses a variety of spectral signatures or multifarious labels to enhance the plex in a limited area.[109] Fluorescence technologies are developed and very sensitive, but subject to autofluorescence and channel bleed-through, and SERS-like methods have sharp spectral characteristics but have substrate reproducibility issues. Decoupling plex and planar area Barcode bead assays coded beads and are then optically or electronically decoded.[110] Despite the fact that this strategy facilitates the ability to achieve higher plex, it involves strong bead handling, mixing and decoding inside a sealed cartridge.[110,111] The use of electrochemical arrays as a point-of-care method is appealing due to the ability to use small and low-cost readers. Table 4 and Figure 6A summarize the major multiplex detection strategies used in exosome nanochips, including fluorescence arrays, electrochemical sensors, plasmonic/photonic sensors, SERS-based platforms, bead/barcode systems, aptamer arrays, digital immunoassays and CRISPR-assisted nucleic-acid readouts. It highlights how different sensing formats support protein- and RNA-based exosome profiling within compact liquid-biopsy devices. Experimental and/or methodological support are indicated for a number of representative systems: for detection based on fluorescence[99]; for SERS/spectral barcoding[100]; for electrochemical affinity biosensing[101]; for FET biosensors[102]; for SPR/LSPR and label-free photonic sensing[86,87,103]; for digital ELISA/single-molecule immunoassays[104]; for bead-based multiplex microfluidics[105]; for DNA-barcoded antibody strategies[106]; and aptamer/oligonucleotide sensor arrays[107]; for CRISPR-assisted nucleic-acid readouts.[108] Representative LoD or sensitivity ranges were shown where available, but as exosome-specific performance is highly dependent on the upstream isolation conditions, matrix background and the performance of the assays, these should be viewed as representative only.

The analysis of sensing modalities reveals that there is no best method if all transition strategies were to be used in every clinical use case. The advantage of platform based on fluorescence is that reagents are well developed and that they are highly sensitive, but also needs optical alignment, spectral correction and control of autofluorescence. Although electrochemical arrays are more suitable for compact point of care readers, electrode fouling, baseline drift and reference-electrode instability start to be significant problems in plasma-rich matrices. Plasmonic and photonic sensors allow for label-free or signal enhanced detection, but are sensitive to changes in refractive index, temperature and differences in the batches of nanostructures. Multiplexing capacity and sensitivity can also be achieved, but the assay becomes more complex, and requiring more reagents and carries a higher risk of contamination associated with digital immunoassays and molecular barcode approaches. Thus, the type of platform should depend on the intended use of the platform: a low-cost longitudinal monitoring platform might favour electrochemical or fluorescence arrays, but complex optical or digital or barcode-based platforms might be more suitable if the use is discovery or confirmatory research.

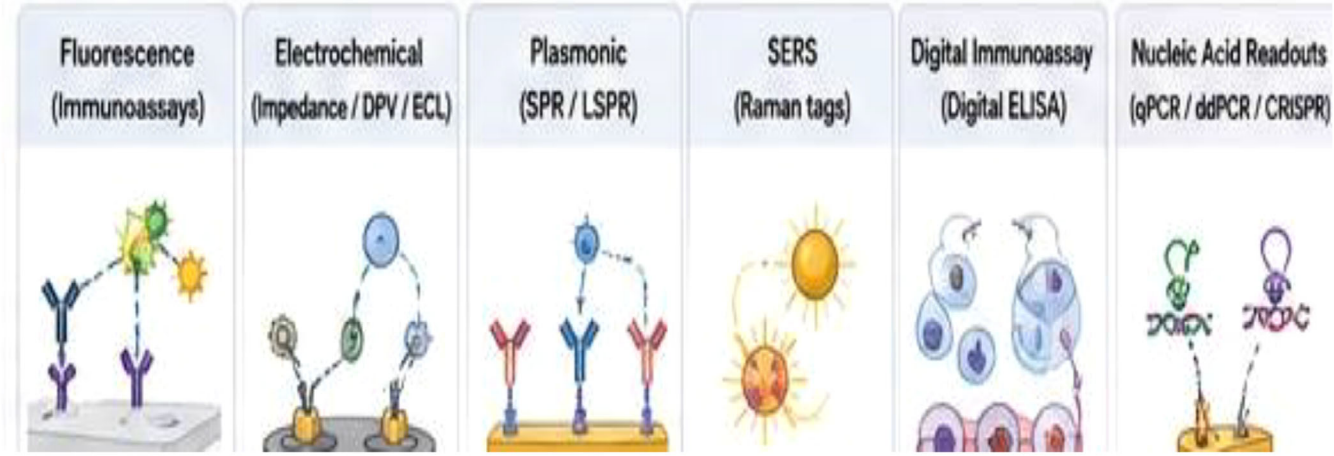
Biological coverage is enhanced and workflow burden has measurable increases with multiplexing. Each added marker may require an additional capture ligand, detection probe, calibration curve, control channel, wash

TABLE 4 Multiplex sensing options for single-drop exosome profiling.

Modality	Typical multiplex strategy	Strengths	Main pitfalls	POC-readiness	References
Fluorescence arrays	Spatial + spectral (multi-spot × multi-dye)	Mature; high sensitivity; wide reagent ecosystem	Optics complexity; autofluorescence; bleed-through	Medium–High	[112]
SERS / spectral barcoding	Spectral multiplex (Raman tags)	Very high plex potential; sharp spectra	Substrate reproducibility; signal hotspots; batch variation	Medium	[113]
Electrochemical arrays	Spatial multiplex (multi-electrode panels)	Compact readers; low cost; easy miniaturization	Fouling; drift; reference electrode stability	High	[114]
FET sensors	Multi-site electronic (sensor arrays)	Ultra-sensitive; integrable electronics; fast	Surface instability in biofluids; fabrication variation; drift	Medium	[115]
SPR/LSPR	Label-free + arrays; kinetic multiplex	Real-time kinetics; no labels	Bulk refractive index shifts in plasma; temperature sensitivity	Medium	[116]
Digital ELISA / single-molecule immunoassays (on-chip)	Compartmentalization (droplets/wells) per target; encoded beads	Exceptional sensitivity; strong clinical precedent	Fluidic complexity; longer workflows; reagent needs	Medium	[117]
Bead-based encoding (fluorescent or magnetic beads)	Bead barcodes (colour/size/shape) + pooled incubation	Scalable plex; concentrates signal; flexible panels	Bead aggregation; decoding errors; nonspecific adsorption	Medium–High	[118]
DNA-barcoded antibodies (immuno-PCR / sequencing readout)	Molecular barcodes per target; pooled readout	Massive multiplex; high sensitivity; small sample	Contamination risk; amplification bias; slower + more steps	Low–Medium	[119]
Aptamer/oligo sensor arrays	Spatial multiplex; sequence-specific capture + signal transduction	Stable reagents; reversible binding; tunable chemistry	Off-target binding in plasma; nuclease sensitivity; calibration burden	Medium	[120]
CRISPR-assisted nucleic-acid readouts (EV-RNA)	Guide-RNA multiplex (sequential or partitioned)	High specificity; isothermal options; strong signal amplification	Sample prep burden (RNA access); multiplex crosstalk; carryover contamination	Medium	[121]

condition, and data-normalization step. Hence, the factors highlighted below should be used together with plex level: Assays per hour, reagent no., zone, reader parts, failed-run rate and cost per test and operator steps. Low-to-moderate panels (3–8 markers) could be more usable for clinical point-of-care applications if designed to maintain total assay time less than 60 min and requiring at most 2–3 manual steps while maintaining predefined LoD/LoQ and inter channel CV < 15%–20%.[122,123] The higher-plex formats like bar code or SERS, or like digital ELISA, or sequencing linked assays may be considered for discovery or confirmatory testing but may ultimately compromise clinical usefulness with increased reagent cost, increased incubation and decoding time, increased calibration burden and increased risk of cross reactivity and channel failure.

However, the electrochemical surfaces are sensitive to foul and drift and thus antifouling strategies, reference channels and occasional calibration methods are required. Label-free or enhanced detection and real-time monitoring



(A)

Detection modality (single-drop nanochip)	Sensitivity	Multiplexing	Matrix tolerance	Reader complexity	Assay time	Fouling / drift risk	POC readiness	Best-fit use
Fluorescence arrays	High	High	Medium	Medium	Medium	Medium	Medium	Protein panels
Electrochemical arrays	Medium	Medium	High	Medium	High	Medium	High	Longitudinal monitoring
SPR/LSPR and photonic sensors	Medium	Low	Medium	High	Medium	High	Low	Label-free kinetics
SERS / spectral barcoding	High	High	Medium	Medium	Medium	Medium	Medium	High-plex profiling
Digital ELISA and bead/barcode assays	High	High	Medium	High	High	Medium	Low	Ultra-sensitive low-abundance targets
CRISPR-assisted EV-RNA readouts	High	Medium	Medium	Medium	Medium	Medium	Medium	RNA-focused assays

(B)

Legend (qualitative rating) High Medium Low Not applicable / lowest

FIGURE 6 (A) Multiplex sensing architectures for exosome profiling. Detection performance depends on upstream exosome enrichment, sample matrix, lysis efficiency, fouling control, optical/electrical background, multiplexing burden and calibration stability under whole-blood or minimally processed plasma conditions. SERS, surface-enhanced Raman scattering; CRISPR, clustered regularly interspaced short palindromic repeats. (B) Performance trade-offs of exosome detection modalities under single-drop whole-blood conditions.

baselines plasmonic and photonic transduction modalities have the potential of facilitating label-free or enhanced detection and real-time kinetic monitoring, but refractive index change by plasma constituents or by changes in temperature can cause perturbation.[124] In modalities, systems with the highest clinical plausibility trade-off reproducibility and interpretability with maximal plex, and use multiplexing to measure the orthogonal biological signals and introduce strong controls instead of increasing panel size. As a matter of fact, both metrics are used to quantify the proportion of target vesicles retained and the proportion of confounding particles that co-isolate in a well-designed study since both measures form the basis of clinical interpretability.[124] At the microlitre level, any small changes in the surface adsorption, valve timing, or wetting of the channels can have a significant impact on yields, so designs should be minimized with respect to the surface interfaces and the wetting properties stabilized.[124] In the case of translational attempts, it is convenient to decouple analytical sensitivity and clinical utility because

a mediocre-sensitive assay can still be of use when it provides predictable trends to track. Where possible, internal standards should be provided at platforms that cross the entire workflow in order to be able to estimate recovery and inhibition per run. Since finger-prick sampling causes a user-dependent variation, strong cartridges usually adopt mechanisms to correct variation in timing, for example, passive metering, clot traps or controlled mixing zones.[125]

Engineering-wise, mechanical or fluidic failures are the most common modes of failure as opposed to subtle biomolecular processes, including bubbles, clogs, incomplete priming and uneven distribution of flow. Finally, manufacturing decisions such as thermoplastic selection, surface activation, reagent storage can tune the performance at the baseline and thus initial prototypes need to expect to scale both materials and processes.[3] In general, credible performance is based on well-designed integrated designs that have transparent controls and standard metrology. Table 3 complements Figure 5 by comparing multiplex strategy, robustness, and reader requirements across major transduction classes.

6.1 | Detection-Platform Selection Under Single-Drop Whole-Blood Conditions

The relative value of each exosome detection modality drastically varies when carried out in a single drop whole blood or a minimally processed plasma format. In this environment, a detector's ability to achieve a low limit of detection without changing its buffer conditions is not the only concern, but its ability to remain specific, reproducible, and interpretable results in the presence of haematocrit fluctuation, haemolysis, platelet activation, lipoproteins as well as plasma-protein fouling, viscosity variation, anticoagulants, and limited sample volume are. Thus, single-drop exosome nanochips should be evaluated based on matrix tolerance, reader complexity, assay time, wash steps, multiplexing burden, calibration stability, compatibility with upstream enrichment and lysis and the capability of incorporation of internal quality-control channels.

One advantage of the fluorescence-based assays is that the reagents used are mature, high sensitivity can be obtained and that spatial and spectral multiplexing can also be accommodated.[126] They are applicable to low to moderate protein panels in single drop cartridges with control of optical alignment, auto fluorescence and spectral bleed-through. The limitations they faced in whole blood applications is that background or optical readout interference is caused by haemoglobin, bilirubin, lipids, cell debris and cartridge materials. Therefore, fluorescence detection is ideally combined with an efficient plasma-separation, plasma washing, reference-spot channel and haemolysis-control channel.[126]

For single drop systems at point-of-care, one of the most practical designs are electrochemical arrays that can be miniaturized, and operated using simple and cheap readers and compact electronics.[4,126] They are particularly suited for longitudinal monitoring panels that may be multiplexed moderately to obtain a fast read-out instead of maximum plex. The use of whole-blood and plasma-rich matrices, however, can result in a significant electrode fouling, unspecific adsorption, redox-background drift, an instability from the reference electrode and signal variation due to conductivity.[4] Thus, the electrochemical exosome nanochip needs to be protected by antifouling coatings, the presence of blank (or reference) electrodes, built-in calibration and matrix compatible washing or dilution procedures.

Label-free detection or signal enhanced detection by plasmonic and photonic methods, such as Surface plasmon resonance (SPR) and localized surface plasmon resonance (LSPR), can also give real-time kinetic information.[127] While convenient for monitoring binding of vesicles to surface markers and binding, whole-blood translation is susceptible to several factors, including bulk refractive index changes, temperature changes, nonspecific protein adsorption and nanostructure batch variation. Plasmonic methods will work best with single-drop cartridges that have upstream matrix clean-up, temperature control, antifouling surfaces and reference channels which will account for the nonspecific refractive index changes.

Although both surface-enhanced Raman scattering (SERS) and spectral-barcoding platforms can be very multiplex, in a clinical sense, they rely on reproducible substrates, stable hotspots, control of nanoparticle aggregation, and reliable spectral decoding in complex matrices for their utility in single-drop testing.[128] Such systems would appear to be more appropriate for confirmatory or research multiplex profiling than for the low cost point of care multiplex analysis without a proven ability to perform substrate reproducibility and automated spectral correction on different lots of chips.

High sensitivity and multiplexing can be accomplished with digital ELISA, bead-based encoding, DNA-barcoded antibody and CRISPR-assisted readouts, especially for the low-abundance protein or RNA cargo.[129,130] But these options may involve other incubation and other partitioning, bead-manipulation, amplification, washing and/or contamination control parameters. These additional steps add to the time of assays in a single drop, the amount of reagent needed, the fluidic complexity and the chance of failure in a single drop closed cartridge. So, they are best suited for high sensitivities and/or high plex

requirements and perhaps simpler fluorescence or electrochemical panels could be best suited to cost effective, expedient, longitudinal monitoring.[130]

In general, there is no single detection modality that is best for single-drop exosome nanochips. Fluorescence and electrochemical platforms generally better suited for quickly multiplexing point of care assays, label free kinetic detection is best served with plasmonic and photonic sensors where robust matrix correction is required; barcode based sensors are suited for higher multiplex assays where in-depth reproducibly control is needed and CRISPR / nucleic-acid amplification assays are suited for assays focussed on RNA when lysis, inhibition control and carryover prevention is integrated into the cartridge. Even for clinical, whole blood use, therefore, the optimal detection platform is one that offers both sensitivity and tolerance to the matrix, along with internal quality control, manufacturability and clinically-interpreted outputs.

Figure 6B compares major exosome-detection modalities according to practical performance criteria relevant to single-drop microfluidic Nano chips, including sensitivity, multiplexing capacity, matrix tolerance, reader complexity, assay time, calibration stability, fouling risk and point-of-care readiness. This comparison helps guide the selection of detection platforms for clinically realistic whole-blood liquid-biopsy applications.

7 | FROM ONE BLOOD DROP TO ANSWER: INTEGRATED SAMPLE-TO-RESULT SYSTEMS

A strict single-drop exosome experiment should include whole-blood manipulation and enrichment, cargo recovery assay, and many measurements but not operator intervention. It should be integrated in that transferring volumes in microlitres between different modules heightens sample losses and moves toward contaminations.[3] Whole blood introduces several constraints: erythrocytes and platelets may obstruct microchannels or generate confounding vesicles, viscosity varies with haematocrit and clotting may occur rapidly in capillary samples if anticoagulation is not controlled.[131] Classifier stability is undermined by even small amounts of haemolysis since it causes the background of proteins and micro-RNA to become destabilized. Thus, the performance limit is set by plasma-handling modules. The massively effective acquisition can be done through inertial microfluidics and membrane-based cell separation is small but subject to clogging and hemolysis.[4] Paper-based microfluidic hybrids can separate cells passively at low cost, and require very strict control of variability. Cartridge automation may be achieved by using capillary valves, dissolvable films, which liberate reagents in accordance to timed elapse, and lyophilized reagents which rehydrate on command.[132] Such features do not only alleviate the variability in the use of the user, but also provide even timing which is critical in the prevention of clots and reproducible binding kinetics. The whole-blood and combined plasma-handling patterns each reveal a comprehensive comprehensiveness in the assay strength and success as depicted in Figure 7. The schematic is grounded in capillary microsampling and finger-prick blood-handling studies,[9,34,47] whole-blood platelet and haemostatic effects,[89] emerging microfluidic plasma-separation technologies,[90] paper-based microfluidic approaches for rapid detection,[91] and EV diagnostic translation/failure-mode considerations.[48] Modules shown as dashed or shaded elements should be interpreted as design requirements that need validation under realistic finger-prick whole-blood conditions.

An endpoint which is clinically interpretable is a requirement before the deployment of assays. Usually, the multiplexed signals are reconfigured back into a classifier or rule bank to produce risk scores/ response categories or longitudinal trends.[133] The system should also raise quality-control concerns, for example, haemolysis, excessive lipoprotein contamination, or non-optimal recovery, and hold back results when internal controls are violated in order to guarantee reliability.

Internal standards should also be put in place within the platform where possible to traverse the entire workflow to estimate run-to-run recovery and inhibition with the goal of doing so. Finger-prick sampling is liable to artefacts due to operators, thus cartridges need to contain a system that reduces timing errors, like passive metering, clot-trap designs or mixing zones. Understanding failure modes On an engineering scale, mechanical and fluidic complications are more common causes of failure as a result of bio-molecular failure; common causes of failure include formation of bubbles, blocking of channels, poor priming and poor distribution of flow.[133] In addition, manufacturing choices, such as the choice of thermoplastic substrate, surface activation methods, and storage format of reagents, can be used to change the baseline performance, and thus require that early prototypes be used to test material and processes that are scalable.

The engineering-based common failure mechanisms are mechanical and fluidic instead of biomolecular, and include formation of bubbles, channel clogging, uncertainties in priming and uneven division of flows. Consequently, the use of single-drop exosome testing can be used with credibility to support robust designs, which use clear controls and standardized metrology, and can thus decentralize clinical decision-making in a wide variety of environments.

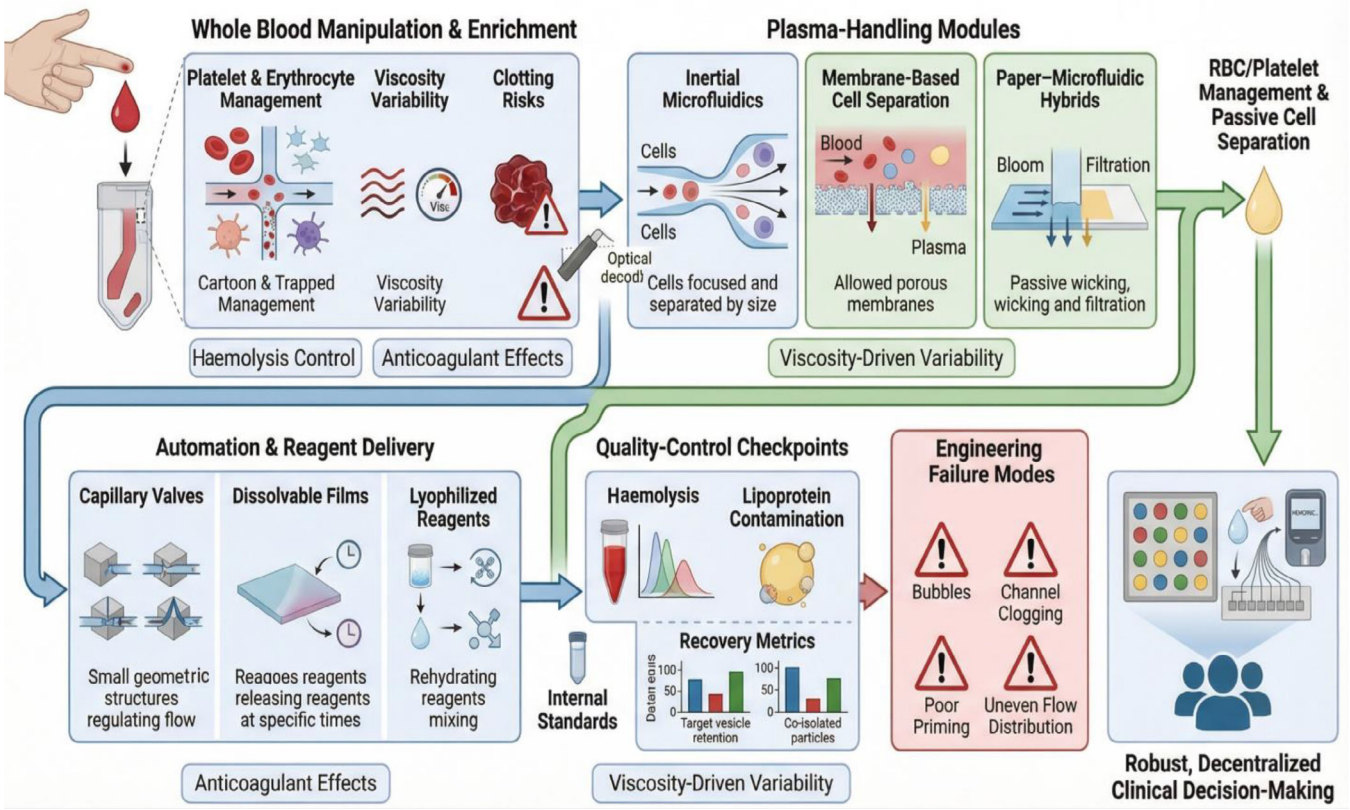


FIGURE 7 Evidence-linked whole-blood constraints and integrated plasma handling for single-drop exosome testing. The figure summarizes blood-matrix and cartridge-level failure modes that influence exosome nanochip performance, including haematocrit-dependent viscosity, platelet activation, haemolysis, clotting, bubbles, clogging, incomplete priming and uneven flow partitioning.

8 | ANALYTICAL/CLINICAL VALIDATION, STANDARDIZATION AND TRANSLATION ROADMAP

One of the biggest issues in the current literature is that current technologies for isolation and detection of exosomes are not standardized and do not have head-to-head comparison. Many studies report excellent performance with optimized buffers, cultured-cell vesicles or pre-clarified plasma, but these do not replicate the confounding effects found in finger prick whole blood such as haemolysis, platelet activation, blood viscosity changes, lipoprotein overlap and the amount of blood available for study. Similar input sample, vesicle standard, recovery and purity definitions and downstream read-out data should be used in future validation studies to facilitate the benchmarking of technologies. If this is not contrasted with others, statements about superiority of any platform, hybrid or otherwise, are hard to assimilate clinically.

The term 'realistic whole-blood validation' should not be confused with blood-derived matrices for simple testing: In this review, clinically meaningful whole-blood validation is used to describe experiments conducted in (usually small-volume) minimally processed fresh and/or appropriately anticoagulated whole blood samples collected at the finger prick or other low-volume scale and then processed in the required amount of samples to operate within the cartridge design. These validation aspects cover all the important pre-analytical and matrix parameters of decentralized testing: haematocrit and viscosity differences, effect of anticoagulants, time delay between sampling and processing, haemolysis, platelet activation, tendency to clot, lipoprotein overlap, nonspecific protein binding and sampling error by operator.[134] Such studies should also indicate if plasma separation, exosome enrichment, access to exosome cargo, detection and data interpretation are all done in the same device or it is still necessary to process disease-carrying cells in another off-chip device.

A variety of studies have shown good analytical performance with either buffer, cultured-cell vesicles, spiked plasma or pre-clarified samples; studies may be valuable in establishing capture chemistry, sensing sensitivity or feasibility of the device; but do not represent the full picture of confounders encountered in finger-prick whole blood.[4,135] Other studies focus on specific aspects of the problem including isolating a specific microsample, separating a plasma sample, capturing the antigen through

immunotargeting, capturing antigen using nanostructured surfaces, or multiplex detecting in a sample; however, none of these studies have completely tested a single drop, sample to answer process under clinically realistic whole-blood conditions.[4] Thus, the degree of translational readiness should be assigned an appropriate level following the degree of matrix realism: Level 1: Buffer standard or purified vesicles; Level 2: Clinical samples with controlled pre-processing spiked with predetermined amount of analytes; Level 3: Venous whole blood with controlled pre-processing; Level 4: Finger prick or low-volume whole blood which has been processed end-to-end in an integrated cartridge with built-in quality-control thresholds.[135] This separation allows the differentiation of analytical sensitivity from clinical utility and is important to understand when considering the need for high analytical sensitivity alone is not enough for clinical translation.

The two major challenges that make striking laboratory prototypes impossible and unacceptable as trustworthy clinical instruments are the analytical validity and reproducibility. Single drop systems should not only be sensitive but also able to recover, be pure and resistant to heterogeneity of whole blood and failed states, due to the small sample volume provided per run which limits repeatability.[136] Validation is to be stratified into separate levels: the first tier is intra-chip repeatability tests to measure the run-to-run accuracy in highly-controlled settings, the second phase is the inter-chip and inter-lot testing to determine the manufacturing variation, and the third level is inter-site checks to evaluate the variation between an operator and an instrument, which is the minimum credible requirement of multicentre clinical investigations.[8] There are required levels of control on each level; spike-in vesicles or synthetic pseudostands measure recovery and can be used to disentangle biological variance with process-related variance; isotype and blank controls are used to define non-specific binding, and lipoprotein and platelet contamination markers are used to argue in support of purity claims.[137] Haemolysis also help in decoding RNA signals and they also eliminate incorrect biological inferencing that occurs due to sample degradation.[137] Clinical validation should be matched with the purpose of use: screening projects demand such large cohort sizes and absolute maximum specificity, and monitoring can be validated through evaluating in-patient trends in accordance with clinically relevant endpoints.

To minimize batch-to-batch variation, exosome nanochips should be manufactured under locked material, surface-activation, antibody-coating, drying and storage protocols, with lot-release criteria defined before use. Each lot should be tested using reference EVs or synthetic vesicle standards, and acceptable performance windows should include recovery CV $\leq$15%–20%, capture efficiency within $\pm$ 20% of reference lots, and stable LoD/LoQ across chips. Consistency across isolation time points should be maintained by fixed anticoagulant type, defined time-to-processing, temperature control, passive metering, clot traps and per-run spike-in recovery controls. Current limitations include lack of universal EV reference materials, limited multicentre lot-validation data and incomplete evidence under finger-prick whole-blood conditions.

The minimum validation and reporting expectation are operationalized in Figure 8 and Table 5, and the translation process is outlined in Figure 9 based on the proposed intended use.

Both standardization and harmonization of reporting allow comparability of studies. Minimal reporting formats should include a description of sample-handling, anticoagulant, time to process, recovery and purity ratios and clear descriptions of training and validation in the use of classifiers.[138] Practically, the most highly informative studies reported to quantify the percentage of the target vesicles retained, and also the percentage of confounding particles co-isolating referred to as such because they deterministically affect clinical interpretability overall.[139]

Where possible platforms ought to combine internal criteria which cut across the entire workflow, and this enables one to estimate recovery and inhibition per-run. Since finger-prick sampling adds variability related to the user, strong cartridges usually allow some means of alleviating sampling timing errors, like passive metering, clot-trapping, or predetermined mixing boundaries. From an Engineering perspective, mechanical and fluidic challenges, such as the generation of bubbles, clogging, incomplete priming, and the uneven distribution of the flow are the most common failure modes, not subtle biomolecular perturbations. Lastly, the process choices in manufacturing (e.g., thermoplastic, surface activation, storage of reagents, etc.) may also change the base operation; hence, initial prototypes must expect scalable material and operations.[140] Practically, the informative studies that will be the most informative will measure the proportion of vesicles of interest retained and the fraction of confounding particles that co-isolate as both determinants are important to clinical interpretability.

Overall, credible decentralization of single-drop exosome testing depends on robust integrated designs with clear controls and standardized metrology. Table 4 operationalizes Figure 8 by listing minimum validation elements and reporting requirements that support comparability and clinical defensibility.

We propose a standardized benchmarking framework for single-drop exosome Nano chips based on five consensus evaluation domains: sample realism, analytical performance, purity/interference control, reproducibility and clinical usability. First, platforms should be tested

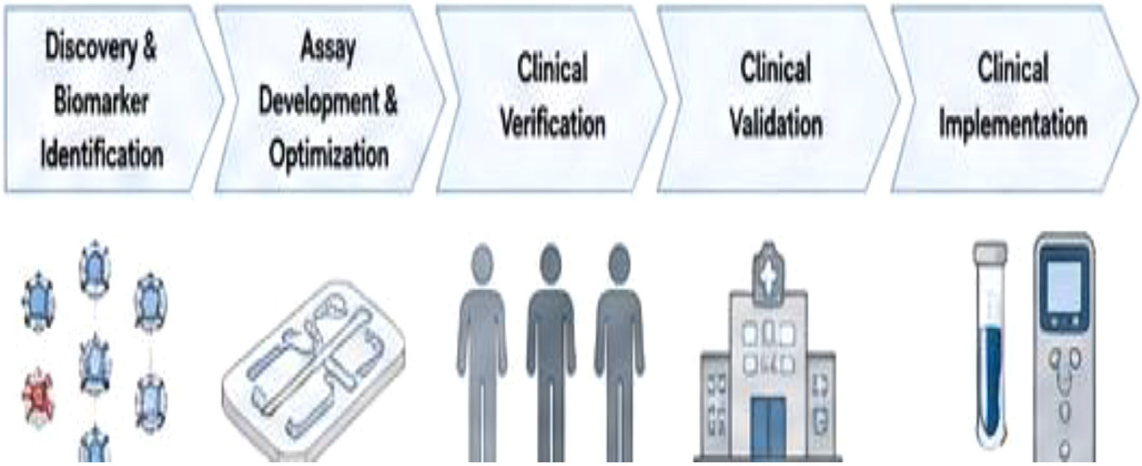


FIGURE 8 Clinical translation roadmap and validation pathway for single-drop exosome nanochips.

TABLE 5 Validation checklist and minimum reporting set for single-drop exosome nano chips.

Category	Minimum requirement	Example metrics/controls
Identity	Demonstrate EV enrichment	CD9/CD63/CD81; sizing (NTA/TRPS); orthogonal confirmation (TEM/cryo-TEM, AFM)
Purity	Quantify confounders	Lipoprotein (ApoA1/ApoB), albumin, platelet markers (CD41/CD61); total protein background; detergent sensitivity
Recovery	Measure yield against input	Spike-in vesicles (synthetic EVs/standard particles); recovery %; capture efficiency vs. input concentration
Analytical performance	Precision + reproducibility tiers	Intra-chip, inter-chip, inter-lot, inter-site CVs; LoD/LoQ; linearity; carryover
Clinical performance	External validation	Independent cohorts; pre-specified analysis plan; ROC/AUC + calibration; subgroup performance
Robustness	Whole-blood stress tests	Haematocrit/viscosity panels; haemolysis thresholds; mixing time/window; clotting tolerance
Stability	Shelf-life evidence	Accelerated ageing; temperature cycling; freeze–thaw; shipping simulation; reagent lot stability
Data integrity	Classifier validity	Calibration + drift correction; bias/fairness checks; leakage prevention; locked model vs. retraining policy
Pre-analytical variables	Standardize collection + handling	Tube type (EDTA/citrate), time-to-test, storage time/temp; agitation; centrifugation-free vs. clarified blood comparators
Interference & specificity	Demonstrate resistance to interferents and cross-reactivity	Endogenous interferents (lipaemia, icterus, haemoglobin); heterophilic antibodies; rheumatoid factor; non-EV particle controls

in matched buffer, plasma, venous whole blood and finger-prick whole blood to define matrix-dependent performance loss. Second, all studies should report recovery, purity, LoD/LoQ, linearity, throughput, assay time and failed-run rate using the same input volume and reference EV or synthetic vesicle standards. Third, confounders should be quantified using ApoA1/ApoB, albumin, haemolysis markers and platelet markers. Fourth, reproducibility should be reported as intra-chip, inter-chip, inter-lot, inter-operator and inter-site CVs. Finally, clinical usability should include number of user steps, time-to-answer, cartridge failure rate, cost-per-test, QC rejection criteria and compatibility with intended clinical use.

9 | CONCLUSIONS

Single-drop microfluidic nanochips are a promising approach to decentralized exosome-based liquid biopsy, but their clinical readiness remains limited. Current platforms have advanced in exosome enrichment, nano-biointerface design, and multiplex detection, yet most

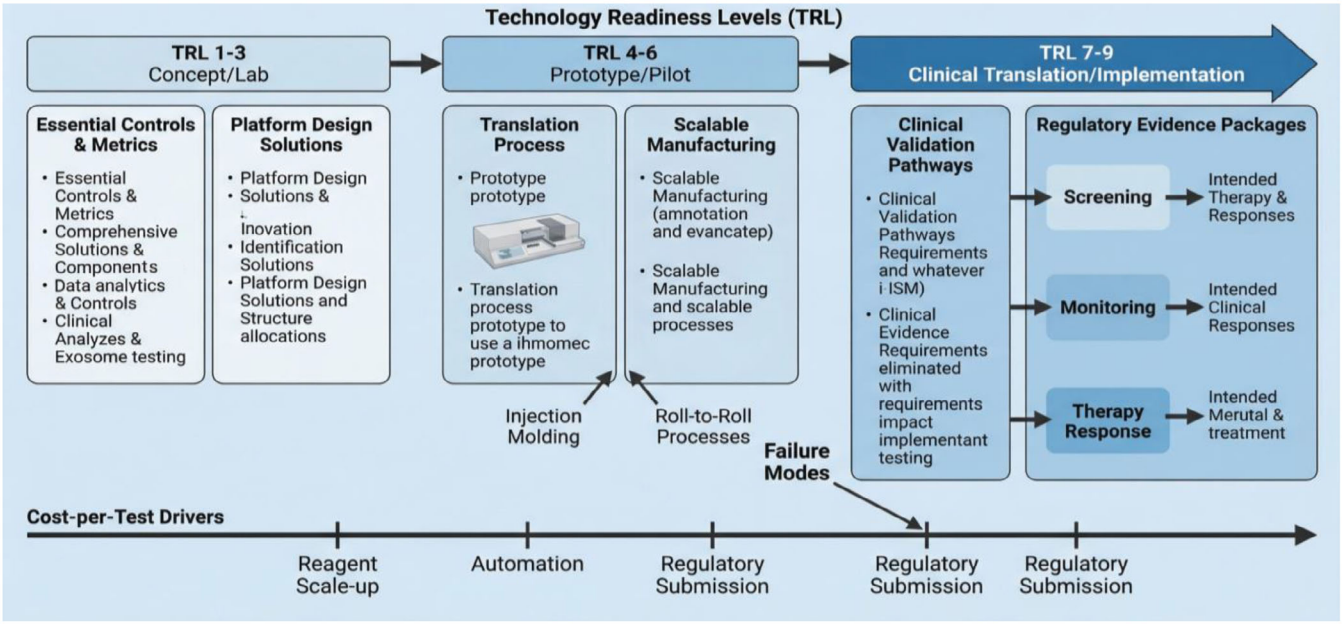


FIGURE 9 Clinical translation roadmap. Technology readiness levels (TRL), manufacturing routes (injection moulding, roll-to-roll), cost-per-test drivers and regulatory evidence packages aligned to intended use (screening vs. monitoring vs. therapy response).

remain proof-of-concept systems rather than fully validated sample-to-answer diagnostic devices. Another area of concern is that much of the research relied on optimized buffers, cultured-cell vesicles, spiked samples or pre-clarified samples of plasma rather than realistically spiked finger prick whole blood, where haemolysis, plate activation, lipoprotein overlap, viscosity variations, clotting and non-specific adsorption effects could result in recovery, purity and signal unreliability.

Also, there is no standard benchmarking of the field. Published studies differ in input volume, the types of vesicles used for standards, the protocol used for recovery calculations, the definition of purity, the detection endpoint, and the reporting of failure modes make it difficult to compare handled studies. Thus, any superiority of any specific strategy, including hybrid platforms, should be guarded until being tested under comparable conditions.

The most critical translational bottleneck in the field is not the lack of highly sensitive exosome sensors, but the lack of reproducible, contamination-aware, end-to-end performance in small-volume whole-blood samples. Microfluidic systems matter because they address a specific unmet need that conventional exosome workflows do not: integration of plasma handling, vesicle enrichment, cargo access, multiplex detection, internal quality control, and automated interpretation within a closed, low-volume cartridge. Therefore, microfluidics should be chosen when the clinical question requires rapid, repeatable, low-sample-volume testing near the patient, especially for longitudinal disease monitoring, treatment–response assessment, minimal residual disease surveillance, or decentralized cohort studies where repeated venipuncture and centralized laboratory processing are impractical. In contrast, conventional technologies such as ultracentrifugation, density gradients, size-exclusion chromatography, nanoparticle tracking analysis, ELISA, RT-qPCR, ddPCR, or high-complexity sequencing remain preferable when the priority is deep molecular characterization, discovery research, large-volume purification, or high-precision reference measurement in centralized laboratories. The real advantage of microfluidics is therefore not merely 'point-of-care use', but the ability to reduce sample volume, shorten time-to-answer, minimize transfer losses, combine orthogonal enrichment and detection steps, incorporate run-level quality controls, and support clinically interpretable trend-based outputs. The translational priority should consequently be the development of standardized, manufacturable, whole-blood-compatible cartridges that demonstrate end-to-end recovery, purity, interference control, and reproducibility across chips, operators, lots and sites.

Because lipoproteins overlap substantially with small EVs in size, density and several biophysical properties, clinically credible single-drop platforms should demonstrate not only exosome recovery but also suppression, depletion, or computational flagging of ApoA1/ApoB-positive lipoprotein-derived signals under lipemic, haemolysed, platelet-rich, and minimally processed whole-blood conditions. Thus, the immediate translational priority is not to maximize analytical sensitivity alone, but to prove that microfluidic exosome Nano chips can deliver reproducible, contamination-aware, clinically

interpretable results from minimally processed whole blood under realistic operating conditions.

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The authors declare no conflicts of interest.

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The authors have nothing to report.

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