REVIEW

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Role of aptamer technology in extracellular vesicle biology and therapeutic applications

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Extracellular vesicles (EVs) are cell-derived nanosized membrane-bound vesicles that are important intercellular signalling regulators in local cell-to-cell and distant cell-to-tissue communication. Their inherent capacity to transverse cell membranes and transfer complex bioactive cargo reflective of their cell source, as well as their ability to be modified through various engineering and modification strategies, have attracted significant therapeutic interest. Molecular bioengineering strategies are providing a new frontier for EV-based therapy, including novel mRNA vaccines, antigen cross-presentation and immunotherapy, organ delivery and repair, and cancer immune surveillance and targeted therapeutics. The revolution of EVs, their diversity as biocarriers and their potential to contribute to intercellular communication, is well understood and appreciated but is ultimately dependent on the development of methods and techniques for their isolation, characterization and enhanced targeting. As single-stranded oligonucleotides, aptamers, also known as chemical antibodies, offer significant biological, chemical, economic, and therapeutic advantages in terms of their size, selectivity, versatility, and multifunctional programming. Their integration into the field of EVs has been contributing to the development of isolation, detection, and analysis pipelines associated with bioengineering strategies for nano-meets-molecular biology, thus translating their use for therapeutic and diagnostic utility.

1. Introduction

Extracellular vesicles (EVs) are membranous vesicles (30–300 nm) released by all cellular organisms, including plants, fungi, and bacteria.[1–4] They act as important signalling entities for the selective packaging and transfer of bioactive molecules such as proteins, lipids and nucleic acids between cells to induce a signalling response and regulate cellular processes.[5] The production and dynamic release of EVs as biological messengers appear to be evolutionarily conserved across all kingdoms of life. Functionally, EVs are involved in different homoeostasis processes, such as metabolism,[6] neuronal function,[7] cell development and differentiation,[8,9] cell proliferation, inflammation,[10] embryo implantation and endometrial function,[11–13] and tissue repair and regeneration.[14–16] EV-mediated crosstalk may occur unidirectionally or bidirectionally between cells or *via* systemic communication, during which EVs traffic to various tissues and organs.[17,18] This intercellular interaction may involve not only the release and delivery of EV cargo but also cell surface interactions and target-cell modulation (*i.e.*, surface-mediated signalling).[19,20] Besides the horizontal transfer of cargo molecules such as proteins and RNAs between cells, EVs can induce signaling in target cells upon interaction at the cell surface (juxtacrine signaling) and complex with and neutralize soluble factors through receptors on the EV surface.[21] Thus, EV membrane proteins, such as proteoglycans, lectins, and integrins, as well as components of the extracellular matrix, mediate the EV–plasma membrane interaction. Moreover, EVs have intrinsic tropism at the tissue and cell levels as a result of their surface displayed molecules. The capacity of EVs to essentially capture a snapshot of selected molecular cargos from their parental cells in space and time and their ability to transfer complex biological information make them unique and promising next generation diagnostics and therapeutics.

EVs may be classified based on a set of characteristics, including their biophysical properties (size and density), bio-

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chemical composition, process of cellular release, and surface charge. As heterogeneously sized (from ∼30 to more than 3000 nm in diameter) particles, EVs include small EVs (sEVs, including exosomes), microvesicles (MVs) (or large EVs (lEVs), microparticles and ectosomes), shed midbody remnants (sMBr), migrasomes and apoptotic bodies[20,22–28] (Table 1). The nature and relative abundance of EV cargo are selectively determined during EV biogenesis,[20,29,30] and depend on their cell of origin[22,31–34] as well as source tissue.[6] In addition, EV cargo loading is influenced by the different physiological, pathological, and stress conditions of the cell/tissue of origin. Over the past decade, including those driven by the international EV community, awareness of the need to rigorously isolate specific EV types and populations for research and therapeutic purposes has been a central focus in the field to better understand their fundamental form and composition, and further elucidate mechanisms of EV biogenesis and function. Currently, size, density, surface charge, membrane surface proteins, and lipid membranes have been explored for the isolation of EVs; specifically, these include differential ultracentrifugation, density gradient ultracentrifugation, polymer-based precipitation, charge-mediated electrostatic adsorption, ultrafiltration, size exclusion, lipid anchors, and antibody-based immunoisolation.[22,46,47] However, there is a pressing need for bioaffinity-mediated EV isolation[48] to capture a disease-subset of EVs, understand a subpopulation of EVs, or even capture cell-specific surface antigens.[31,34,49,50] Innovation in the development of novel technologies for cell- or tissue-specific targeting of EV will accelerate the clinical translation of EV-based diagnostics and therapeutics. Recently, the development of aptamer technology has seen multidisciplinary applications and impacts on various aspects of the field of EVs, including enrichment and retrieval, biology and signalling, imaging, targeting, and therapeutic delivery. Their facile modification and programmability as well as their significant affinity recognition ability to target and interact make the feature of aptamers a single-stranded DNA sequence highly advantageous[51] in promoting the understanding, targetability and therapeutic enhancement of EVs.

2. Therapeutic extracellular vesicles

EVs have inherent advantages as therapeutic mediators due to their size, membrane stability, non-toxicity and non-immunogenicity, and propensity for cellular uptake and transversion of biological barriers, including tissue barriers (blood–brain, blood–cerebrospinal fluid barriers), plasma membranes, and their ability to deliver cargo across endosomal membranes. In addition to these intrinsic properties, the ability to modify the surface and luminal cargo loading using various molecular biology and bioengineering approaches makes EVs an attractive vehicle for delivering a wide range of therapeutics.

EV-associated bioactive cargo has been shown to promote several processes in the body that contribute to endogenous tissue remodelling and therapeutic tissue repair. Stem- and progenitor cell-derived EVs have been shown to mediate therapeutic effects in various preclinical disease models, which have resulted in the initiation of several early phase clinical trials.[52,53] Moreover, pre-clinical studies on cardiac pathology highlight the regenerative capacity of EVs and engender their use as cell-free therapeutic tools.[54] Clinical applications of EV-based therapeutics, derived from various immune cells (e.g., dendritic cells[55–59] and engineered cell counterparts[60–64]), tumour cells,[65–67] and bacteria,[68] are being trialled in immunomodulation in the context of antigen cross-presentation, cancer immune surveillance and tumour escape, tissue regeneration and recovery, and as delivery vehicles for combination therapies.[50]

In addition to the delivery of bioactive cargo, EVs harbor various cell surfaces and integral membrane proteins based on their source, mediating associated cell recognition, adhesion, antigen uptake, and recipient cell function.[69–74] In addition to target cell surface interactions, EVs can also bind complement

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Dr Chowdhury is an early career researcher in cancer diagnostics, specializing in aptamer-based techniques. With a Bachelor's and a Master's in Microbiology, and a PhD in Molecular Medicine, he currently works as a Research Fellow at Laboratory of Aptamer Medicine at Deakin University. His work focuses on developing aptamer-based methods for isolating and detecting cancer-derived extracellular vesicles in blood samples, as well as using aptamer-based constructs for sensitive cancer biomarker detection in tissue biopsies.
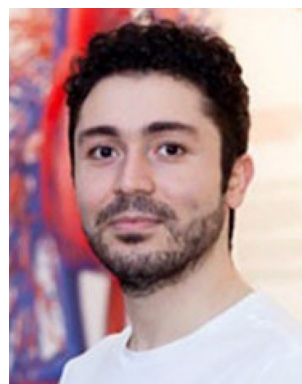
Sadegh Eslami

Mr Eslami is a PhD candidate in the Molecular Proteomics group and holds a bachelor's degree in biological science and master's in Medical Biotechnology. His research interest in extracellular vesicles (EVs) has led him to understand the therapeutic application of these vesicles, their engineering and regenerative capacity in cardiac biology. His current studies focus on defining their signalling and function in overcoming the therapeutic challenges associated with cardiovascular disease, and developing next generation therapeutic strategies

factors and complement regulatory proteins,[19,75] as well as cytokines,[76] with such surface composition a functional determinant of EV–cell interactions, impacting the accumulation of EVs in specific cell subsets and organs.[76] Immune (dendritic) cell-derived EVs, harbouring functional membrane-bound IL-15Rα (NKG2D ligand), were shown *in vivo* to promote receptor–ligand-dependent immune cell activation and proliferation, resulting in tumour suppression.[58] EVs from monocyte-derived dendritic cells also carry functional NKG2D ligands and can promote the proliferation and activation of NK cells,[58] suggesting their potential utility for tumour vaccination.

In the context of tissue regeneration and repair, stem cell-derived EVs can ameliorate the effects of various diseases in the liver (fibrosis, hepatitis, and inflammation), brain (stroke), heart (myocardial infarction and contractility), kidney (renal ischemia and stenosis), and immune system. Moreover, EVs secreted from differentiated induced pluripotent stem cells, cardiospheres or embryonic stem cells administered directly into infarcted hearts have been shown to attenuate ischaemic injury in both small and large animal models.[77,78] Through direct administration to the heart, EVs are believed to signal directly to the myocardium and supporting cells, including fibroblasts and endothelial cells, altering their responses to ischaemic injury. The diverse beneficial effects of seemingly identical entities (*i.e.*, mesenchymal stem cell (MSC)-EVs, and bone marrow-derived stem cell (BMDSC)-EVs[79–84]) suggest a complex repertoire of active cargo[85] working synergistically, as opposed to a single molecular component. Applying a system-based understanding of EV form and effect, such as omic and multi-omic-based approaches, is currently a focus in the EV field.[86,87]

Current advances in the field of EV therapeutics have highlighted current and emerging strategies in engineering EVs (either through altered cell-based genetic engineering, or directly altering EV form/type through EV modification) as an effective means to tune a specified function for an intended disease phenotype. Various strategies to genetically engineer cells to produce EVs with a protein tag for the targeted delivery of biologics to immune cells without the need for chemical modifications have been shown.[88] Furthermore, EVs provide a deliverable approach to ablate protein expression (*i.e.*, through the delivery of the programmable nuclease Cas9); alternately, co-delivery of a homology-directed repair template can insert specified mutations, insertions or deletions into the genomes of target cells, or further through EV-mediated delivery of adeno-associated virus (AAV) vehicles to overcome anti-vector immunity and inherent limited tissue tropism. Cells genetically engineered to produce functionalized EVs may even be implanted to continuously generate such particles *in situ*. In contrast, although modification of EVs directly may confer cargo loading flexibility, this approach requires more extensive purification and introduces challenges from a manufacturing and regulatory standpoint.

Recently, aptamer technology was applied to the field of EVs based on specific interactions between aptamers and EVs; such studies have highlighted their potential associated with EV isolation, the capacity to modify and enhance various interactions between aptamers and EVs to understand specific EVs, and cargo packaging in EVs for delivery and gene editing.

3. Aptamer: a unique class of nucleic acid-based affinity ligands for biology and medicine

Aptamers are short single-stranded nucleic acid molecules with the ability to fold into defined three-dimensional struc-
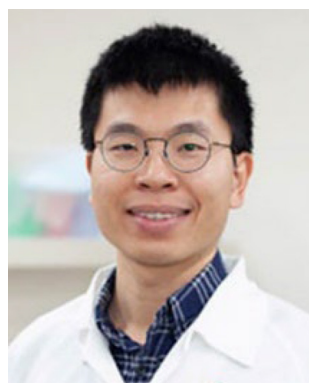
Cuong Viet Pham

Dr Pham completed his PhD in Medical Biotechnology in Oct 2022 at Deakin University. His PhD projects focused on cancer liquid biopsy through the development of novel analytical methods that can sensitively and specifically quantify and classify cancer biomarkers for cancer-derived extracellular vesicles. He is currently working on engineered extracellular vesicles along with next-generation mRNA for personalised medicine in Molecular Imaging and Theranostics Laboratory at the Baker Institute. With expertise in nanomaterials, nanotechnology, cell biology and biochemistry he aims to developing novel theranostic technologies for cardiovascular diseases.

Alin Rai

Dr Rai is an expert in the functional role of extracellular vesicles (EVs), including exosomes, and signaling biology, quantitative proteomics, transcriptomics, bioinformatics and systems biology. Within the Molecular Proteomics team, his research focuses on developing and applying quantitative proteomic techniques to study the molecular function of EVs, and quantitative analysis of dynamic protein abundance of normal physiology and disease-perturbed signalling networks associated with cardiometabolic disease. In addition to extensive student supervision, he has led the development of novel approaches to understand EVs from human circulation, identifying new therapeutic targets for clinical intervention, and establishing commercial partnerships.

tures and thus can bind specific targets.[89] Also known as "chemical antibodies", aptamers have a distinct molecular identity as well as a disparate route of selection from their protein counterparts, antibodies. Being DNA or RNA, aptamers are selected *via* an *in vitro* PCR-based procedure termed the systematic evolution of ligands by exponential enrichment (SELEX), which was developed in 1990.[90,91] Unlike the production of antibodies, which rely on the use of animals or cultured cells, SELEX is conducted in a microtube without the involvement of animals or cells. Therefore, aptamers have been selected against small inorganic molecules, proteins, specific types of cells or organs, and even whole organisms with high specificity and affinity.[92–95] Because of the *in vitro* nature of selection, aptamers can be developed with higher affinity for their cognate targets (K_D of 10^{-12} M) than for antibodies[96] with comparable specificity. Therefore, there is increased interest in adopting aptamer technology in both basic biomedical research and sustained efforts in its translation into the clinic (Fig. 1).

As nucleic acid-based affinity ligands, aptamers have several advantages over antibodies in their applications in biomedical research and clinical translation.[97] Being chemically synthesized, aptamers have very low and controllable batch-to-batch variations in terms of the quality and consistency of the synthesized DNA or RNA. They can also be rapidly and scalably generated in large quantities. The stability of DNA and chemically modified RNA aptamers is unrivalled by antibodies, as aptamers remain stable in low or high pH, in an organic solvent, over a sustained period of elevated temperature, and can withstand physical forces, *e.g.* shaking or vortexing. Pertaining to EV research and development, several distinct features make aptamers an invaluable class of affinity ligands. First, most aptamers possess a molecular mass of 10–15 kDa,

which is one order of magnitude smaller than that of monoclonal antibodies. Being small, the motion of aptamers is much faster than antibodies in aqueous milieus *in vitro* and *in vivo*, as the speed for a molecule moving *via* Brownian motion is inversely related to its size. Second, a monoclonal antibody of 150 kDa has a coverage footprint area in the range of 38–88 nm^2,[98] while that for an aptamer of 15 kDa is in the range of ~8–16 nm^2.[99] This translates to 10-fold more molecules of aptamer binding to the same area occupied by a single monoclonal antibody on the surface of the EV membrane. The mechanism underlying the advantage of more aptamers engaging an area occupied by a single antibody has been elucidated in an elegant study on the selectivity of targeted drug delivery, where Prins and co-workers have shown that multivalent weak interactions between DNA ligand and its receptor on particles lead to enhanced selectivity.[100] Consequently, the small size of aptamers affords enhanced binding avidity and specificity of aptamers to their targets on the surface of EVs. Third, aptamers bind to their targets *via* their properly folded three-dimensional structure *via* induced fitting[101] without the involvement of covalent bonding. As shown in Fig. 2, the binding motif of aptamers is generated by folding the linear DNA or RNA strand *via* internal hydrogen bonding into a defined 3D structure. Unlike antibody-based affinity chromatography in which harsh conditions are employed to elute the bound antigen, *e.g.* extreme pH and high salt, the bound antigen or EV-associated (surface) ligand can be released readily from aptamer *via* the disruption of the 3-D confirmation of the aptamer under physiological conditions. Finally, as per molecule, a DNA aptamer is generally 50–100 times cheaper than a monoclonal antibody. Thus, it is commercially uncompetitive, if not unviable, to use antibody-based chromatography to manufacture large quantities of
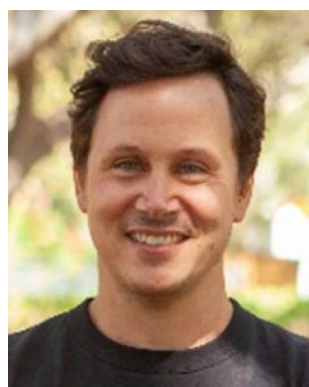
David W Greening

A/Prof. Greening employs a multi-disciplinary approach to understand the molecular function of extracellular vesicles incorporating proteomics, lipidomics, cell biology, molecular biology, and physiology, with the goal of identifying new deliverable therapeutic targets. His team employs advanced quantitative mass spectrometry (MS) to understand proteome regulation and unravel the mechanistic insights of complex biology and biological systems. He leads the Molecular Proteomics group, and Research Platform at the Baker Institute. His internationally recognised research has led to important developments in delivery-based therapeutic applications for various metabolic and cardiac diseases & adopted internationally for clinical trials through extensive research engagement and translation.

Wei Duan

Professor Wei Duan obtained his Ph.D. in Biochemistry and Molecular Biology at University of Melbourne in 1991. Following his postdoctoral fellowship at University of Melbourne, he took a faculty position at the Medical School of National University of Singapore. He joined Deakin University in 2006, where he is currently an Alfred Deakin Professor at Deakin School of Medicine. Prof. Duan's research focuses on developing novel aptamer-based technologies for biomedical applications. He has published over 260 original research papers and review articles with an h-index of 64. He was 2017 Clarivate Analytics Highly Cited Researcher in Pharmacology/Toxicology.

Table 1 Subtypes and bio-characteristic properties of extracellular vesicles

	Extracellular vesicles			Other types of extracellular vesicles			
Subtype	Small EVs (sEVs)[23,33,35–39] (incl. exosomes)	Large EVs (lEVs)[23,29,40,41] (incl. ectosomes, microvesicles)	Shed midbody remnants (sMB-Rs)[26]	Exopher[42]	Large oncosome[43]	Migrasomes[28,44]	Apoptotic bodies[45]
Biogenesis	Endosomal maturation pathway, intraluminal vesicles (various sorting mechanisms)	Plasma membrane budding or protrusions, membrane fission	Cytokinesis (cytokinetic abscission)	Plasma membrane budding	Plasma membrane budding	Plasma membrane budding	Plasma membrane budding
Markers (enrichment)	CD63, CD81, SDCBP, ESCRT complex proteins: ALIX/ PDCD6IP and TSG101	ANXA1, RPS7, ARF6	MKLP1, RACGAP1	Phosphatidylserine, LC3, Tom20	CK18, GOT1	TSPAN4, ITGA5	Phosphatidylserine
Particle diameter range	30–200 nm	~100–1000 nm	~200–600 nm	≥1 μm	≥1 μm	≥1 μm	50–5000 nm
Buoyant density	1.08–1.14 g ml^{-1}	1.08–1.14 g ml^{-1}	1.22–1.30 g ml^{-1}				

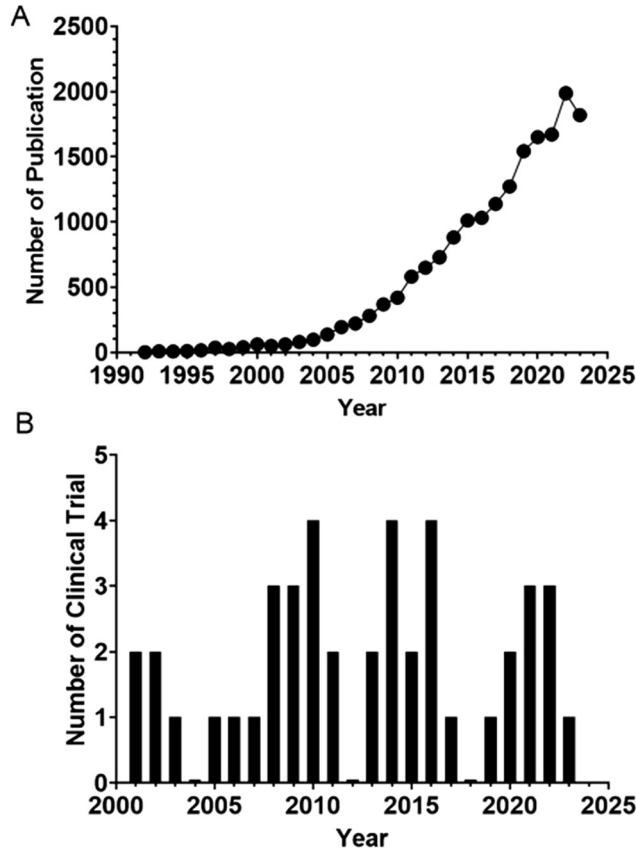**Fig. 1** (A) Counts of yearly publications on aptamer in PubMed. (B) Number of clinical trials per year in NIH clinical trial registry (https://clinicaltrials.gov/).

biotherapeutics, such as EVs. In addition, the pharmaceutical industry routinely uses alkaline solutions, *e.g.* 0.1 M NaOH, to clean or sterile chromatography vessels. These alkaline solutions totally destroy the antibody's ability to bind to their targets. In contrast, DNA aptamers can withstand 30 cycles of treatment with 1 M NaOH, followed by washing with saline without compromising their binding capacity.[102] Needless to say, extreme pH and high salt inevitably damage the structural integrity and functional competence of EVs isolated *via* affinity chromatography. Since 2010,[103] aptamers have been applied as a frontier technology for EV biology and research, including their application in isolation, detection, labelling/tracking and EV-based diagnostics and therapeutics (Fig. 3, Tables 2 and 3).[51,89]

The transition from bench to clinic presents significant challenges, with the efficacy of aptamers reliant on understanding the role of their targets in disease pathology. Moreover, the decision to utilize aptamers as either standalone treatments (monotherapy) or as part of a multi-agent regimen in clinical trials profoundly influences the outcome. Table 4 provides a summary of the clinical trials involving 15 classes of aptamers over the past decade, sourced from ClinicalTrials.gov. Among them, ten aptamers successfully completed Phase 1 trials but did not progress to Phase 2. Additionally, three aptamers concluded Phase 2 trials
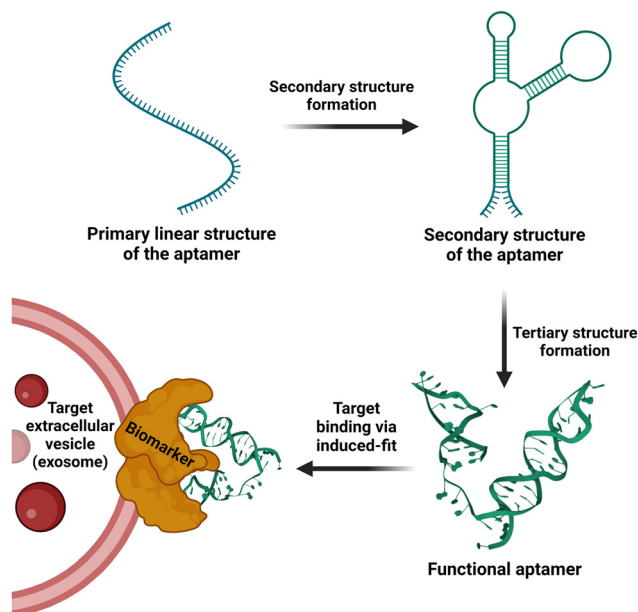
Fig. 2 Schematic representation of folding of an aptamer from the initial linear strand into a defined 3D structure followed by target binding.
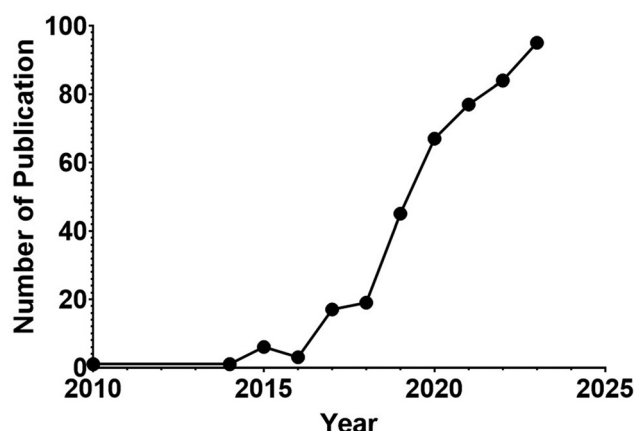

Fig. 3 Counts of yearly publications on application of aptamer technology in EV research in PubMed.

without advancing to Phase 3, which is primarily attributed to their inability to meet the primary endpoints. Finally, one aptamer's Phase 3 trial failed due to its lack of efficacy. Remarkably, the anti-complement C5 RNA aptamer, avacincaptad pegol, has persevered through a 15-year clinical development journey. It transitioned from multi-agent combination trials to a single-agent monotherapy trial, ultimately achieving FDA approval in September 2023. This milestone came 15 years after its initial Phase 1 trial in 2008.[135] Avacincaptad pegol now stands as the second aptamer approved by the FDA, offering treatment for geographic atrophy secondary to age-related macular degeneration.[136]

4. Aptamer-based sensor for the detection and analysis of EVs

Many aptamers developed previously for cell biology and cancer biology have been utilized for the detection and analysis of EVs,[89,137] including aptamers against PSA, HER2, PD-L1, CEA, VEGF, PSMA, and EGFR. One of the most frequently utilized aptamers in the EV field is a 32-nucleotide DNA aptamer against human CD63 with a K_D of 40 nM developed by Base Pair Biotechnologies, Inc. (#ATW0056).[138] Various aptamer-based biosensors (aptasensors) have been developed with modes of signal transduction, including fluorescent, electrochemical, colorimetric and surface-enhanced Raman spectroscopic detection.[139] The signal of aptamer-based sensors can be further enhanced *via* direct amplification in which nucleic acid amplification products are generated on the EV surface or competitive amplification; aptamers competitively bound on EVs can release trigger DNA upon interaction between aptamers and their targets on EVs for subsequent amplification. The progress on aptasensors over the past decades has been extensively covered in recent reviews.[139–141] Here, we discuss new developments since 2023. Li and co-workers developed an aptamer-based method for the detection and classification of ovarian cancers by profiling tumor markers on small EVs.[142] They isolated EVs from plasma by ultrafiltration, followed by the analysis of a set of seven EV surface cancer biomarkers, *i.e.* CA125, STIP1, CD24, EpCAM, EGFR, MUC1, and HER2, using a nanoflow cytometer, a flow cytometer purposely developed for the analysis of EVs from 40 to 1000 nm in size and equipped with single-photon counting capacity.[143,144] By molecular profiling plasma sEVs from 54 patients with untreated ovarian cancer, they were able to identify patients with cancer with an accuracy of 94.2%. Huang *et al.* developed an electrochemical biosensor with divalent aptamer-functionalized nanochannels by simultaneously conjugating CD63 and EpCAM aptamers.[145] The target EVs can be recognized and selectively captured in a divalent collaborative manner, resulting in a varied ionic transport behaviour corresponding to the abundance of captured EVs. This method achieves an impressive limit of detection (LOD) of 4.43×10^3 particles per mL with HeLa cell-derived EVs in 10 mm KCL (pH 7.4). A similar dual aptamer recognition strategy was employed by Guo and colleagues who constructed a fluorescent biosensor using DNA aptamers against CD63 and PTK7, achieving a limit of detection (LOD) of 2.2×10^7 particles per mL using EVs from MCF7 breast cancer cells.[146] Li and colleagues also employed a CD63 aptamer to develop a molybdenum disulfide-integrated iron organic framework.[147] This is a colorimetric aptasensor with a mimetic nanozyme (MoS_2-MIL-101(Fe)) with superior peroxidase enzymatic activity to generate a change in colour in the TMB-H_2O_2 system to report the detection of EV, with an LOD of 3.37×10^6 particles per mL when studied using EVs derived from the HGC-7901 cell line. Furthermore, Cheng and colleagues developed a clove-like gold nanocluster aptasensor in which a CD63

Table 2 The application of aptamers for EV isolation, detection and labelling/tracking

	Target	Aptamer	Source of EVs	Affinity (K_D)	Applications	Ref.
Isolation	CD63	CD63 aptamer	MDA-MB-231, A549 cell culture medium	38.71 nM to CD63	Magnetic bead-based exosome immuno-separation system	104
	HepG2	LZH8	HepG2 cell culture medium	96 nM to HepG2	A nanotetrahedron (NTH)-assisted aptasensor for direct capture and detection of hepatocellular exosome aptamer-based magnetic isolation	105 and 106
	MUC-1	MUC-1 aptamer	Plasma from patients with cancer or healthy subjects	NA		107
	PTK7	Sgc8	A549 cell culture medium	NA	Microfluidic system	108
	Vascular endothelial growth factor	VEGF aptamer	EV from serum of patients with prostate cancer	400 nM	Using nano-flocytometry-based thermophoretic profiling of EVs for the early detection and classification of prostate cancer	106 and 109
	PSMA	PSMA	EV from SKBR3 and LNCaP cells and blood of cancer patients	NA	Multiplex detection of cancer EVs *via* surface enhanced Raman scattering (SERS)	110
Detection	MUC-1	S1.3/S2.2	Exosome-derived from MCF-7, SGC7901 cells, and serum from patients with breast and gastric cancer	0.135 nM to MUC-1 peptides	Electrochemical, fluorescence, colorimetric, and SERS based aptasensors	111 and 112
	HER2	HER2 aptamer	Exosome-derived from HCC1954, P0403, HEK-293, A549, HCT116, SK-BR3, HeLa, MCF-7 cells, and human plasma	NA	Colorimetric, SPR, and SERS based aptasensor for specific HER2 + exosomes detection	111
	αvβ6	αvβ6 aptamer	Exosome-derived from HCC1954, P0403, HEK-293, A549, HCT116, SK-BR3, HeLa, MCF-7 cells, and human plasma	NA	Colorimetric, aptasensor for specific αvβ6 + exosomes detection	113
	PD-L1	MJ5C	Exosome-derived from A375 and k562 cells and human plasma.	91 ± 12 nM to PD-L1 positive cell	HOLMES-Exo_PD-L1 fluorescent sensor for detection of glycosylated PD-L1 exosomes	114
	ALPPL-2	SQ2	Exosome-derived from PANC-1+, Capan-1, cells, and human serum.	22.5 ± 4.5 nM to ALPPL-2	Colorimetric sandwich-based sensor	115
	CEA	CEA aptamer	Exosome-derived from MCF-7, SK-BR-3, MDA-MB-231, BT47 cells and human serum.	NA	Magneto-ediated electrochemical and SERS based sensor	111
	PSA	AS2	Exosome-derived from PC3, HeLa cells, and prostate cancerous and human plasma.	0.7 nM to PSA protein	Label free qualitive and quantitative aptasensor	116
	PDGF	Group 36	Exosome-derived from HepG2, HeLa, MCF-7, MCF-10A cells, and human blood.	0.58–78 nM to different isoforms of PDGF	ExpoAPP-fluorescence-based aptasensor	117
	PSA	PSap4#5	Human serum	40 nM	Using fluorescent aptamers to discriminate prostate cancer from benign prostate enlargement *via* thermophoretic profiling	106 and 118
	EpCAM		CF-7 cell-derived exosomes in bovine serum	NA	A colorimetric aptasensor to detect and quantify cancer cell line-derived sEVs	119 and 120
	Mycobacterial EV membrane	Aptamer-21	Mycobacterial EV	65 nM	Visualizing the release of mycobacterial membrane-derived EVs in infected macrophages using confocal microscopy	121
	Alkaline phosphatase placental-like 2	SQ2	Blood EV from cancer patients	23 nM	Using ALPPL2 directed sandwich aptamer-linked immobilized sorbent assay for the detection of pancreatic cancer-derived EV	115 and 122
Tracking and labeling	PD-L1	MJ5C	Exosome derived A375 human melanoma cells culture medium	91 ± 12 nM to PD-L1 positive cell	Aptamer-based metabolic glycan labeling for *in situ* visualizing and functional study of exosomes	123
	ATP	ATP aptamer	Exosome derived HepG2 cells culture medium	NA	Aptamer-based probing of exosome internalizing pathways *via* confocal microscopy	124
	CEM cells	Sgc8	Exosome derived immature dendritic cells culture medium	0.8 ± 0.09 nM to CEM cells	Tracking of cellular uptake pathway of aptamer-functionalized exosomes	125
	PD-L1	Ex-50.T	EV from HeLa, Cal27 and 293FT cells	NA	Detecting PD-L1 positive sEV on magnetic chips	126

Table 3 Summary of therapeutic application of aptamers for EVs

Aptamer	Target and disease	Therapeutic cargo	Source of EVs	Ref.
AS1411	Nucleolin. Breast cancer, leukemia, and colorectal cancer models	cel-miR-67 and let-7 miRNA, miRNA-21 Sponge and doxorubicin	Immature dendritic cells, recombinant 293T cells, HEK-293 cells	127 and 128
A9g	PSMA. Prostate cancer cell lines and mouse model of prostate cancer	siRNA against survivin	HEK-293 cells	129
EGFRapt	EGFR. Breast cancer cell lines and mouse model of breast cancer	siRNA against survivin	HEK-293 cells	129
sgc8	PTK7. T-leukemia cell line	Doxorubicin	Immature dendritic cells	125
5TR1	MUC-1. Breast cancer cell line and mouse model of colorectal adenocarcinoma	Doxorubicin	Mesenchymal stem cells	130
A9g	PSMA (K_D 130 nM). Prostate cancer xenograft	survivin siRNA	HEK293T	129
LJM-3064	Myelin. Suppression of inflammatory response as well as lowered demyelination lesion region in CNS.	MSC-derived exosomes	MSC-derived exosomes	131 and 132
BMSC-specific aptamer	Bone marrow stromal cells. Promoting bone regeneration in a mouse model of ovariectomy-induced postmenopausal osteoporosis.	Bone marrow stromal cell-derived exosomes	Bone marrow stromal cell	133 and 134

aptamer-modified DNA tetrahedron was attached to the surface of a gold electrode. Following EV-CD63 aptamer interaction, a sequential DNA aptamer against mucin 1 (MUC1) in a clove configuration was used to generate a strong electrochemical signal, resulting in an LOD of 1.58×10^5 particles per mL using MCF7 cell-derived EVs.[148]

In terms of clinically relevant sensitivity, many aptasensors possess an LOD sufficient for detecting the lower end of the concentration range of the analyte in the targeted sample matrix. For example, the LOD for SARS-CoV-2 detection using an electrochemical aptasensor is <1100 copies per mL,[149] while for a capacitive laser-induced graphene-based aptasensor, it stands at 1790 copies per mL.[150] These LODs translate to a detection capability that is 2 orders of magnitude beyond the acceptable diagnostic sensitivity for SARS-Cov-2 (1.0×10^6 genome copies per mL) according to the World Health Organization.[151] As for protein detection, an on-chip integrated graphene aptasensor achieves an LOD of 160 aM for COVID-19 neutralizing antibodies in serum,[152] which is 5 orders of magnitude lower than the minimum target concentration (30 pM) in patients.[153] Thus, the current generation of aptasensors can attain the sensitivity and specificity required for real-world applications.

5. Aptamer as the next generation affinity ligand for EV isolation

Over the past five decades, the technologies used for the isolation and separation of EVs have harnessed the biophysical properties of EVs, including charge, buoyant density, solubility and size.[48] However, the emergence of biochemical interaction-based affinity isolation methods provides enhanced isolation of EV from cells, biofluids, and tissue sources.[154,155] Interestingly, as revealed by a survey of methods used for the isolation of EV in the last two decades, affinity isolation was the least utilized method employed by the EV field; for example, 8% of published papers used affinity isolation com-

pared with 46% of publications that used ultracentrifugation and 18% papers that used size exclusion chromatography.[156] Despite unparalleled purity and high EV recovery rates, the adoption of affinity chromatography for EVs is hampered by several inherent limitations, including high cost in the use of monoclonal antibodies and limited scalability. As antibody-focused approaches are the most widely used class of ligands in EV affinity chromatography, the harsh elution conditions (non-physiological pH and high salt) required to release EVs from the antibody matrix are key factors that discourage the utilization of affinity-based methods for EV isolation.[157–159]

It is in this context that aptamers emerge as the next generation of alternative affinity ligands for the isolation, concentration and purification of EVs under physiological conditions. The fundamental biochemical and structural differences between antibodies and aptamers lie in the fact that the former consists of 20 monomer units (amino acids) with a very stable 3D structure, while the latter comprises only 4 monomer units (nucleotides) with a 3D structure that can be facilely altered after the capture of EVs to release EVs under physiological conditions. This inconspicuous attribute of aptamers is best exemplified by the pioneering work of rapid capture, followed by the non-destructive release of EVs.[107] Zhang and co-workers conjugated a biotinylated CD63 DNA aptamer to streptavidin-coated magnetic beads. Upon capture of EVs derived from the conditioned culture medium of MCF-7 cells, the EVs were released in phosphate-buffered saline at 37 °C for over 15 min by the addition of 1.5 μM full-length antisense oligonucleotide. This approach altered the 3D structure of the folded CD63 aptamer, abolishing its ability to bind to CD63 on the surface of EVs (Fig. 4).[70] More innovative aptamer-based affinity EV isolation strategies have been developed in subsequent years. Travas-Sejdic and colleagues developed an electrochemically switchable cloth by conjugating a thiolated CD63 aptamer to AuCl4− on a microporous cloth. The captured EVs were released with up to 92% efficiency under −1.2 V voltage in 5 min (ref. 160) although the CD63 aptamer remains attached to the surface of the EVs.

Table 4 Clinical trials for aptamer (2014–2023)

Aptamer	Target	NCT number	Conditions	Year started	Study status
AON-D21	Complement component C5a	NCT05962606	Community-acquired pneumonia	2023	Recruiting (phase 2)
AON-D21	Complement component C5a	NCT05018403	Phase 1, first-in-man single ascending intravenous doses	2021	Completed (phase 1)
AON-D21	Complement component C5a	NCT05343819	Phase 1, multiple ascending intravenous doses	2022	Completed (phase 1)
X-aptamer	Biomarkers for hepatocellular carcinoma patients treated with Lipiodol TACE	NCT04459468	To identify proteomic biomarkers for outcome prediction of TACE Treatment in liver cancer	2022	Recruiting
ApTOLL	Toll-like Receptor 4	NCT05293236	COVID-19	2022	Phase 1 terminated due to lack of patients
AptameX	S Protein of SARS-CoV-2	NCT04974203	Saliva-based COVID-19 test,	2021	Unknown
Tenofovir (TFV) aptamer	Tenofovir (TFV)	NCT04870671	Biosensor for TFV in biological fluid	2021	Completed (phase 1)
BT200	A1 Domain of Von Willebrand Factor (VWF)	NCT04677803	Von Willebrand diseases; Hemophilia A	2020	Completed (phase 2)
Aptamer against antiretroviral drugs	Dolutegravir, emtricitabine, lamivudine, tenofovir	NCT04302896	Urine test for adherence to antiretroviral drugs	2020	Completed (early phase 1)
Zimura®/Izervay® (Avacincaptad pegol)	Complement component C5	NCT04435366	Geographic atrophy; macular degeneration	2020	Phase 3, completed in 2023, FDA approved
ApTOLL	Toll-like Receptor 4	NCT04742062	Stroke	2019	Completed (phase 1)
68Ga-Sgc8	Protein tyrosine kinase-7 (PTK7)	NCT03385148	Colorectal cancer	2017	Unknown (early phase 1)
Zimura® (Avacincaptad pegol)	complement component C5	NCT05571267	Macular degeneration	2016	Phase 2, completed
Aptamer against atrial fibrillation biomarkers	Atrialf fibrillation biomarkers	NCT03188484	To Identify proteins differentially expressed in atrial fibrillation using SOMAscan® platform	2016	Phse 1 completed in 2019
Fovista® (E10030)	Platelet-derived Growth factor (PDGF)	NCT02387957	Age-related macular degeneration (AMD)	2016	Phase 2 terminated in 2017, primary endpoint not achieved
Zimura®/Izervay® (Avacincaptad pegol)	Complement Component C5	NCT02686658	Geographic atrophy; macular degeneration	2016	Phase 2; completed in 2019
Aptamer against bladder cancer biomarkers	Bladder cancer biomarkers	NCT02957370	Aptamer sensors for clinical staging and monitoring of bladder cancer	2015	Completed in 2022
Zimura® (Avacincaptad pegol)	Complement component C5	NCT02397954	Idiopathic Polypoidal Choroidal Vasculopathy	2015	Phase 2 completed in 2019
Fovista® (E10030)	Platelet-derived growth factor (PDGF)	NCT02214628	Age-related macular degeneration (AMD)	2014	Phase 2 terminated in 2019, primary endpoint not achieved
Fovista® (E10030)	Platelet-derived growth factor (PDGF)	NCT01940887	Age-related macular degeneration (AMD)	2014	Phase 3 terminated in 2017, primary endpoint not achieved
Spiegelmer lexaptepid pegol (NOX-H94)	Hepcidin	NCT02079896	Anemia in end stage renal Disease	2014	Phase 1 and 2 completed in 2015
Fovista® (E10030)	Platelet-derived growth factor (PDGF)	NCT02591914	Neovascular age-related macular degeneration (AMD)	2014	Completed (phase 1)

Alternatively, Qian *et al.* developed a dual aptamer-based microfluidic approach to isolate EVs by conjugating the desthiobiotin-labelled CD63 aptamer and PTK7 aptamer onto the surface of a microfluidic chip functionalized with streptavidin. Because the binding affinity of desthiobiotin to streptavidin is five orders of magnitude lower than that for biotin,[161] Qian and colleagues were able to elute up to 10^8 EVs per ml in 20 min with a large amount of free biotin. Once again, both the CD63 and PTK7 aptamers remained on the surface of the EVs. In an attempt to further enhance the efficiency of EV-aptamer-based affinity isolation, a multivalent DNA flower strategy has been pursued.[162,163] To achieve a multivalent interaction between the capture aptamer and target proteins on the EV surface, a single copy of the DNA aptamer against

EpCAM or PTK7 was converted to a long single-stranded aptamer with up to 800 repeated units by enzyme-catalysed rolling circle amplification. The biotinylated long aptamer repeats were then self-assembled into microspheres in a one-pot reaction. The DNA flower-captured EVs were released *via* the digestion of the DNA flower with deoxyribonuclease I. This multivalent affinity isolation method is robust and highly efficient, with a small caveat of contamination of the eluted EVs with deoxyribonuclease I (Fig. 5).

One of the limitations of aptamer-based affinity isolation of EV is the presence of co-eluent in the isolated EV, *i.e.* antisense oligonucleotides in the case of CD63 aptamer-based EV isolation or DNase I and biotin-oligonucleotide in the case of isothermal enzymatic isolation. Depending on the nature of
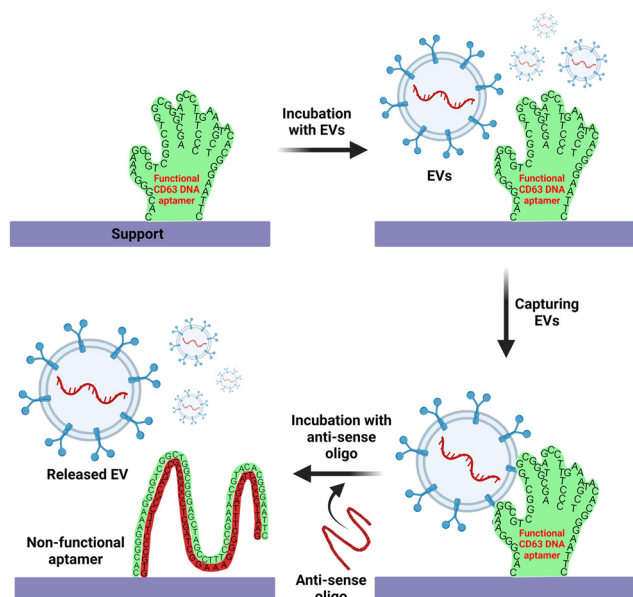
Fig. 4 Schematic illustration of affinity isolation of EV using CD63 DNA aptamer followed by the releasing of EVs in physiological conditions. Adapted from ref. 107.

downstream applications, there is flexibility in how one handles isolated EVs. For instance, in omic-based studies, one may choose to utilize EVs with co-eluted contaminants. Alternatively, one can implement an extra purification step to eliminate these co-eluted substances efficiently. This may be accomplished by employing either streptavidin magnetic beads/columns or size exclusion chromatography to ensure the preservation of the integrity of the isolated EVs.

6. Application of aptamer technology in EV-based translational medicine

6.1 Aptamer-functionalized EV therapeutics for nanomedicine

Since the first approval of PEGylated liposomal doxorubicin (Doxil®) as a nanomedicine by the US Food and Drug Administration in 1995, many new classes of nanoparticle-based nanodrug systems have been developed for improving treatment efficacy and reducing toxicity *via* improved drug solubility, optimized biodistribution and controlled drug release.[164] Moreover, the utility and advantage of nano-medicine as nanosized formulations permit pharmacokinetic and biodistributions that are different from those possible for
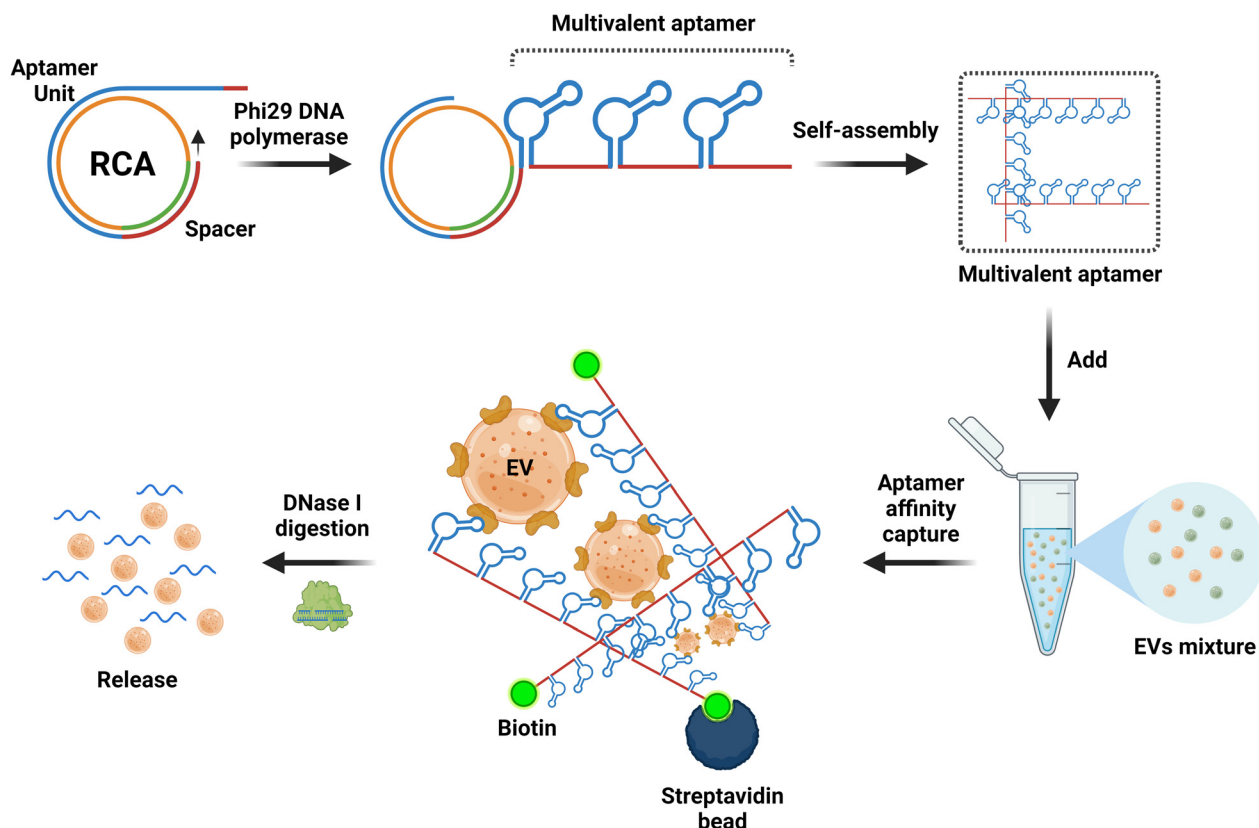

Fig. 5 Schematic of the multivalent aptamers prepared by an isothermal enzymatic techniques known as rolling circle amplification (RCA) for aptamer-based EV isolation. Biotin-modified multivalent aptamers with up to 800 repeated aptamer units are prepared by RCA, followed by the formation of a 3D aptamer network in a suspension for the aptamer-based affinity isolation of EVs. The EVs are retrieved by degradation of the aptamers with dna nucleases. Adapted from ref. 161 and 163.

free drugs and hence can confer considerable therapeutic benefits.[18,165] In particular, functionalized nanodrug systems can offer enhanced bioavailability of free drugs, prolonged half-life of systemically administered drugs, reduced immunogenicity, and targeted delivery to specific tissues.[165]

To complement these systems, EVs have emerged as a promising nano-sized drug delivery platform due to their structural stability, low immunogenicity, human compatibility and degradability and enhanced safety emanating from their acellular nature[18] (Table 5). Therapeutic cargos, e.g. nucleic acid, protein, small molecular drugs, can be loaded into (or surface modified on) EVs by pre-loading methods in which cells are co-incubated with cargos or transfected with target genes, followed by the production of EVs or post-loading where isolated EVs are loaded with cargos via electroporation, sonication, freeze-thawing or chemical induction.[166,167] However, EVs cannot generally target specific types of cells or tissues; thus, it is desirable to modify and enhance the intended therapeutic effect of EVs by targeting moieties for precision medicine.[51]

The aptamer is an ideal class of targeting moiety for EV-based therapeutics because it is vastly superior to its antibody counterpart in tissue penetration and can deliver cargos selectively to target cells in vivo.[168,169] Aiming at the isolation of aptamers that specifically recognize EVs from breast cancer cells, Esposito and colleagues developed an Exo-SELEC procedure in which EVs from breast cancer cells were used to select aptamers targeting breast cancer EVs.[170] The group isolated a 2′-fluoro-pyrimidine modified RNA aptamer termed ex-50.T of 33 nucleotides in length with a K_D of 0.8 nM towards EVs from breast cancer cells. This ex-50.T aptamer was able to inhibit EV uptake by MDA-MB-231 cells by ~50% and inhibited MCF-7 cell migration induced by MDA-MB-231-derived EV by 30%. Thus, this novel aptamer against breast cancer-derived EV provides a strategy for cancer specificity and EV targeting.

The presence of mRNAs and miRNAs in EVs was first reported by Valadi and co-workers in 2007.[174] Since then, EV-based delivery of therapeutic RNA into target tissues has been an area of intense investigation in translational medicine.[175] However, most efforts have focused on EV-mediated delivery of miRNA because the RNA cargos in native EVs are largely small RNAs. The size of mRNA is much larger (1000–1500 nt); therefore, it remains a major challenge for mRNA as a cargo for EV-mediated therapeutic delivery. In an attempt to develop an intradermally delivered mRNA for collagen-replacement therapy, You and co-workers loaded collagen mRNA into EVs from human dermal fibroblasts via cellular nanoporation for the treatment of photoaged skin.[176] In this brute de force approach, transient nanometric pores are created on the surface of cells to allow for the large-scale loading of full-transcript mRNAs into secreted EVs by applying transient electrical pulses of ~200 V.[177] However, a more elegant and potentially widely applicable strategy for loading EVs with mRNA of interest is derived from the utilization of our knowledge of the biogenesis pathways of EV for therapeutic cargo loading.[178] Recently, Zhang and colleagues developed an EV biogenesis pathway-based molecular engineering strategy to load long mRNA into EVs using a DNA aptamer for gene therapy.[179] Based on an unorthodox hypothesis that translation inhibition of target mRNA is beneficial for sorting into EVs, Zhang et al. designed a special DNA aptamer consisting of a single-strand segment matching the region surrounding the start codon AUG of the target mRNA and a double-stranded segment that could be recognized by zinc finger motifs (Fig. 6). They first engineered host HEK293 cells by overexpressing a recombinant protein via the fusion of CD9 with a zinc finger motif. By design, the single-stranded segment of the aptamer prevents ribosome assembly in the AUG and thus arrests the translation of the target mRNA. At the other end of the aptamer, the double-stranded segment binds to the zinc finger fused with CD9, thus shuttling the target mRNA into the endosome to generate EVs loaded with the mRNA. They demonstrated that large mRNA with 2382 nt could be successfully loaded into EVs using this approach. To explore the potential of thera-

Table 5 Bio-characteristics of extracellular vesicles, synthetic nanoparticles, polymers, and free drugs

| Characteristic	Extracellular vesicles	Liposome/synthetic nanoparticles	Polymer	Free drug
Size	40–150 nm (dependent on subtype)	25–1000 nm	50–1000 nm	1–5 nm
Source	Natural cells	Liposome synthesis	Chemical synthesis	Chemical, peptide synthesis
Structure	Phospholipid bilayer, comprise proteins, lipids, and nucleic acids	Double-layer membrane, comprise fat-soluble molecules	Polymerization, comprise polymers	Peptide, enzymes, small molecules
Complexity	++++	++	++	+
Toxicity	Low	Low	High	Low
Stability	High	Low	High	Varied
Multifunctionality (modified, diverse purpose)	+++	+++	+	+
Immunogenicity	+	++	++++	++
Targeting capacity	Inherent/modifiable	Modifiable	Modifiable	Limited
Circulation time	++	+++	+++	+

Adapted from ref. 171–173.
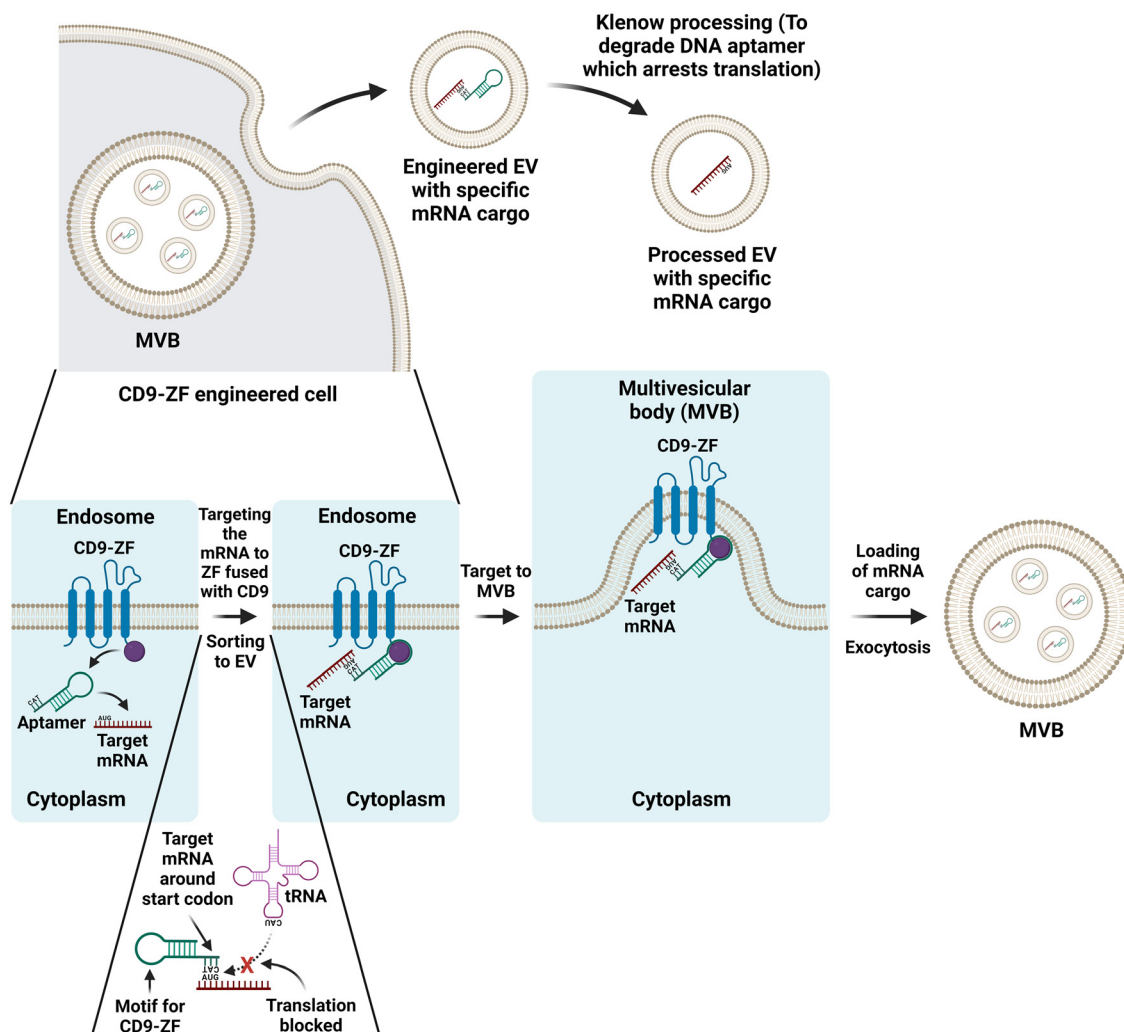
Fig. 6 Schematic illustration of selective sorting of target mRNA into CD9-zinger finger (ZF) engineered EV by a dna aptamer. Top: the therapeutic mRNA is targeted to MVB followed by the loading of the mRNA into EVs by the transfected DNA aptamer within cells engineered by overexpression of the recombinant CD9-ZF. Finally, the DNA aptamers in the released EVs are enzymatically removed. Bottom: the translation of the target mRNA is arrested by the annealing of the single-stranded segment of the DNA aptamer to the regions surrounding the start codon AUG of the target mRNA, followed by the targeting of the mRNA to the endosome membrane *via* the interaction between the double-stranded motif on the DNA aptamer and the ZF in the recombinant CD9-ZF protein. Eventually, the EVs loaded with the target mRNA are formed at MVB and released by exocytosis. Adapted from ref. 179.

peutic applications, Zhang and co-workers further demonstrated that EVs loaded with *Pgc*1α mRNA induced adipose tissue browning *in vivo*, while EVs loaded with interlukin-10 mRNA suppressed intestinal inflammation in a mouse model of inflammatory bowel disease.[179] With further refinement, this EV biogenesis-based mRNA loading strategy will accelerate a new frontier for EV-based mRNA therapy, including novel mRNA vaccines.

6.2 Aptamer-guided EV drug delivery into the brain

Gliomas make up virtually 80% of all lethal primary brain tumours, of which glioblastoma multiform (GBM) is the most malignant type with a 5-year survival rate of <10%. The 15-month average life expectancy of GBM patients has remained unchanged over the past several decades largely due

to the invasive nature of tumour growth and the hindrance of the blood–brain barrier (BBB) in treatment efficacy.[180] Compared to synthetic drug delivery systems, *e.g.* polymeric nanoparticles and liposomes, EVs have low toxicity, excellent biocompatibility, high stability and favourable penetration of the BBB.[181] Aptamers are often employed as a targeting moiety for targeted delivery into the brain. For example, EVs from naïve macrophage can cross the BBB *via* the interaction between integrin lymphocyte function-associated antigen 1 (LFA-1), intercellular adhesion molecule 1 (ICAM-1) and the carbohydrate-binding C-type lectin receptors expressed on endothelial cells on the BBB.[182] Therefore, Liu and co-workers surface-functionalized EVs derived from macrophages with a cholesterol-modified AS1411 DNA aptamer against nucleolin, a protein that is overexpressed on the plasma membrane of

some tumor cells. The AS1411 aptamer-decorated EVs were then loaded with glutathione-responsive biodegradable silica nanoparticles and injected intravenously. After crossing the BBB and entering the tumour cells *via* endocytosis, the EVs produced large amounts of O_2 to relieve tumour hypoxia using an orthotopic GBM xenograft model. Such aptamer-EV mediated sonodynamic therapy efficiently inhibited the GBM metastasis *via* the activation of HIF-α1. A different approach was adopted by Ye and colleagues, who generated aptamer-guided EVS encapsulating two chemotherapy drugs.[183] They initially loaded EVs derived from the human leukemia monocytic cell line THP-1 with temozolomide and O^6-benzylguanine, followed by the conjugation of two targeting ligands to the surface of drug-loaded EVs, an angiopep-2 peptide for enhanced BBB penetration and a CD133 RNA aptamer for targeting the cancer stem cells of GMB. After intravenous administration, the enhanced BBB penetration and superior tumor accumulation of the engineered EVs were prominent in a U87MG-bearing glioma mouse xenograft model, with a therapeutic efficacy 60 fold higher than that of the free drugs.

Parkinson's disease is a progressive neurodegenerative disease that affects a person's control of their body movements.[184] The vast majority of PD subtypes and most cases of sporadic PD share Lewy bodies as a characteristic pathological hallmark, with α-synuclein aggregates being the main component.[185] In pursuing a reduction in neuropathological deficits, Zheng and co-workers developed DNA aptamers targeting α-synuclein aggregates.[186] These aptamers can effectively reduce α-synuclein aggregation in a neuronal cell line and primary neurons and reduce mitochondrial dysfunction and cell defects induced by α-synuclein overexpression. Subsequently, the DNA aptamer F5R1 with a K_D of 2.4 nM was found to bind preferably to fibrillar rather than monomeric α-synuclein.[187] Subsequently, the therapeutic potential of F5R1 was investigated by encapsulating the aptamer into EVs from HEK293 cells following polyethylenimine-assisted transfection. To facilitate the penetration of the BBB, they modified the aptamer-loaded EVs by fusing the neuron-specific rabies viral glycoprotein (RVG) peptide to the extra-EV N terminus of Lamp2b, an abundant membrane protein of EV, to allow facile entry into the brain. Intraperitoneal administration of the RVG-decorated EVs containing the F5R1 aptamer to C57BL/6J female mice led to retrograde transport and transsynaptic transmission into the central nervous system through the axons and synapses of peripheral neurons, circumventing the BBB.[187] Such EV-aptamer treatment decreased α-synuclein aggregation in the substantia nigra and ameliorated motor dysfunction in the mouse α-synuclein preformed fibril model of the sporadic Parkinson's disease. This pioneering work provides valuable insight into the potential for the application of EV-DNA aptamer therapy delivered into the brain for the future treatment of neurodegenerative diseases.

6.3 Aptamer-targeted EVs in regenerative medicine

Regenerative medicine applies engineering and life science principles to repair or replace damaged tissues, promote cell survival and tissue/organ renewal and restore normal functions.[188] Due to their ability to self-renewal and differentiate into specific cells, stem cells are frequently employed to regenerate and repair tissues that have been damaged or affected by the disease.[189] Mesenchymal stem cells or mesenchymal stromal cells (MSCs) are adult stem cells that encompass a broad differentiation potential. In the past few decades, MSCs have been isolated and characterized from various human tissues (*e.g.*, bone marrow, fat, placenta, and umbilical cord) and have been applied in regenerative medicine.[190] Various key studies have highlighted that the paracrine signaling factors of stem cells (and not the cells directly) reduce injury resulting from myocardial ischemia/reperfusion;[191] this foundation has now indicated that many, if not most, of the reported effects of MSC are actually mediated by soluble factors released by these cells, in particular the EVs released by MSCs.[192,193] Intense interest and efforts have expanded this field using EVs from MSC in the development of novel therapeutic strategies for regenerative medicine. However, the risk of an off-target effect due to the low efficiency of EV delivery to the site of injury, in particular the specific type of cells underlying the pathology, remains a major barrier to the successful translation of EV-based therapy. The employment of aptamers to guide EVs loaded with therapeutic cargos to the site of tissue/organ injury has become an increasingly attractive strategy for developing translational strategies for regenerative medicine.[194]

The critical importance of targeting MSC-derived EVs by an aptamer to the intended sites of clinical intervention was demonstrated by Luo and colleagues.[134] These researchers found that EVs from bone marrow stromal cells could effectively augment the osteoblastic differentiation ability of bone marrow mesenchymal stem cells (BMSCs) *in vitro*. However, they failed to prevent ovariectomy-induced postmenopausal osteoporosis using a mouse model. To mitigate this issue, a 40 nt DNA aptamer targeting BMSC[133] was conjugated on the surface of EVs. As expected, such aptamer-guided EVs were able to be efficiently internalized into BMSCs *in vitro* and accumulate in bone marrow *in vivo*. Furthermore, in the bone, the aptamer-targeted EVs could promote bone formation and enhance bone mass in mouse models of postmenopausal osteoporosis and femur fracture *in vivo*.[134]

The indispensable role of aptamers in targeting EVs in the development of efficient strategies for the prevention and treatment of osteoporosis and fracture was further independently confirmed by Shou and colleagues.[195] Zhou *et al.* utilized a three-way junction RNA nanoparticle to engineer EVs functionalized by the same 40 nt DNA aptamer used by Xie *et al.* for bone-specific targeting. As shown in Fig. 7A, they began by linking the 40 nt DNA aptamer against BMSCs to one of the arms of the three-way RNA scaffold,[196] followed by the conjugation of a cholesterol molecule to the 2nd arm of the RNA junction with a triethylene glycol (TEG) spacer for anchoring onto the membrane of EVs. To enable tracking of the aptamer-functionalized EV *in vivo*, a fluorescent reporter (Alexa Fluor 647) was conjugated to the last arm. This BMSC aptamer was
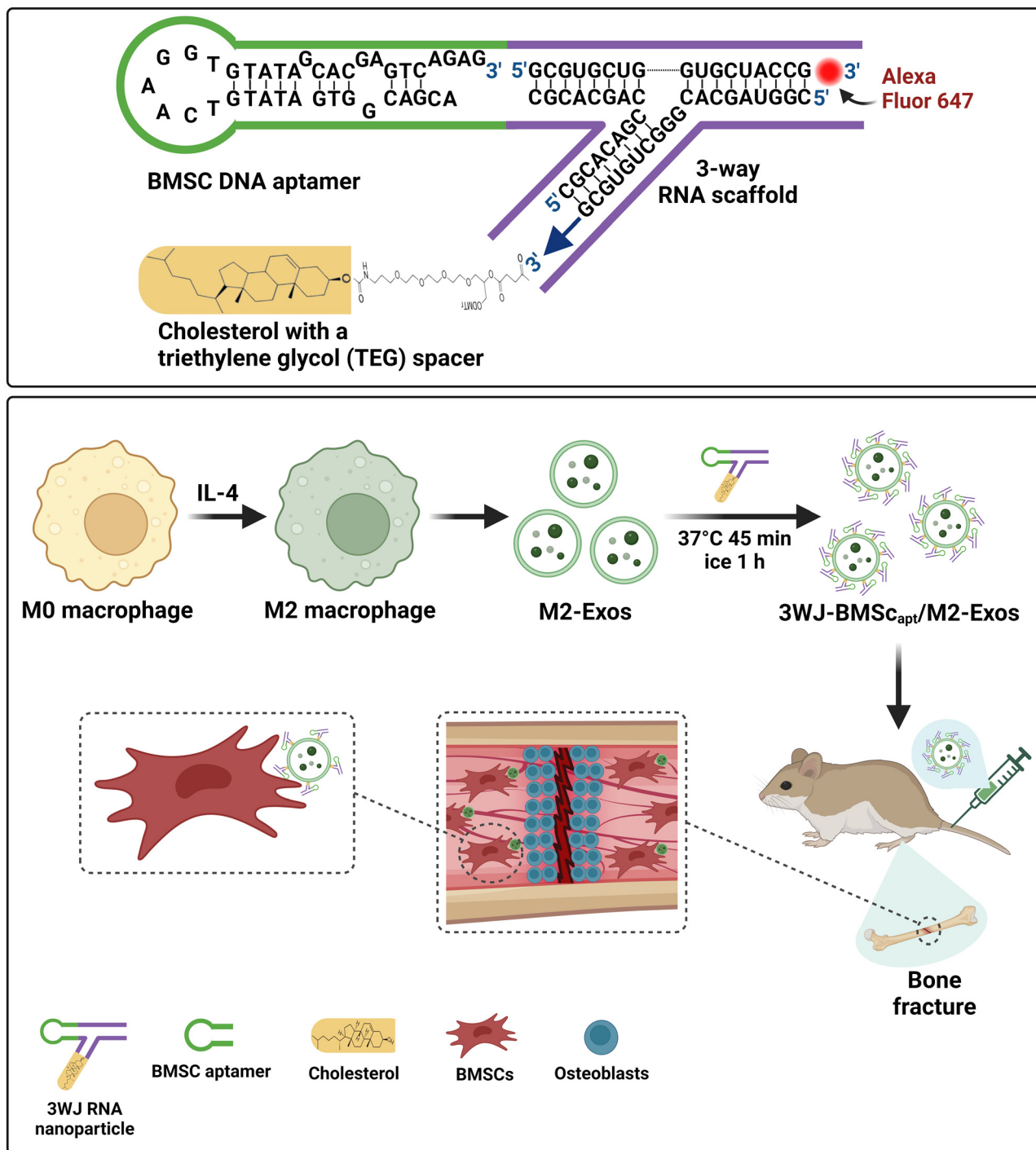
Fig. 7 Schematic illustration of promotion of the healing of bone fracture by 3-way junction RNA nanoparticles–DNA aptamer functionalized EVs. (A) The formation of the 3-way junction RNA nanoparticles by linking of the DNA aptamer targeting BMSC to one arm of the 3-wyar RNA junction and the conjugation cholesterol to the other end of the RNA junction for the insertion of the nanoparticles onto the membrane of the EVs derived from M2 macrophages. A fluorophore is conjugated on the third end to enable *in vivo* tracking. (B) The DNA aptamer-guided EVs are injected intravenously to mice from a femur fracture model. These BMSC aptamer functionalized EVs are targeted to the site of bone fracture followed by a slow release of miRNA cargos, thereby significantly accelerating the bone regeneration processes. Adapted from ref. 195 and 196.

used to functionalize the surface of EVs derived from M2 macrophages that had been shown to attenuate inflammation and promote tissue repair, most likely *via* EV cargos of miR-690 and miR-378a-3p.[197,198] The targeting of aptamer-EV

to BMSCs *in vitro* was confirmed by flow cytometry and confocal microscopy analysis. The DNA aptamer-guided EVs were then systematically administered through tail vein injection to mice from a femur fracture model. These BMSC aptamer-func-

tionalized EVs were found to accumulate mainly at the site of bone fracture with a slow release of cargos, thereby significantly accelerating the healing processes compared with that of the control EVs without BMSC aptamer (Fig. 7B). This work provides an elegant illustration of how aptamer-guided EVs can circumvent the limitation of unmodified EVs, as the latter are rapidly accumulated in and removed by reticuloendothelial organs, *e.g.* the liver and spleen, upon systemic administration.[199] Notably, the utilization of the three-way junction approach for functionalizing EVs with a targeting aptamer exemplifies the versatility of the aptamer armamentarium, as such an approach can be adapted for inserting any targeting aptamer of interest into the membrane of any type of EVs relevant to the intended basic research or pre-clinical purpose.

An alternative way to conjugate aptamers to the surface of EVs is to covalently conjugate a carboxylic acid-functionalized aptamer to the amine groups on EVs through 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)/ *N*-hydroxysuccinimide (NHS) chemistry. Using this approach, Shamili and co-workers functionalized EVs from MSC with a 40-nr DNA aptamer LJM-3064.[132] Because the LJM-3064 aptamer has a high affinity for myelin and the ability to promote remyelination, it was employed as both a targeting ligand and a therapeutic agent. When intravenously injected into mice with experimental autoimmune encephalomyelitis, the LJM-3064 aptamer-EV produced a robust suppression of the inflammatory response as well as a lowered demyelination lesion region in the central nervous system, resulting in reduced severity of the disease *in vivo*.

6.4 Aptamer-guided EVs as a novel therapeutic modality for cardiovascular diseases

Cardiovascular diseases, including hypertension, heart failure, atherosclerosis, stroke, and ischemic heart disease, are the leading causes of death worldwide.[200] EVs have emerged as a promising treatment strategy for cardiovascular disease. As a vehicle for signalling molecules, EV-mediated communication between different heart cells has been shown to play a crucial role in the maintenance of cardiac homeostasis and the progress of cardiac diseases.[201,202] With the advantages of stability, minimal immunogenicity and the ease of pre- and post-isolation modification, several MSC-derived EV therapeutic studies have been developed with therapeutic cargos consisting mainly of microRNAs as well as long non-coding RNA and covalently closed RNA.[203] EV-mediated delivery of miRNAs, including miR-19, miR-210, miR 133a, miR 30d-5p, miR-214-3p, let-7i-5p, let-7g-5p, miR-129-5p, miR-320a, miR-1246, miR19a, miR-29b, miR-455, miR-17 and miR-210, have been explored for EV-mediated treatment of coronary artery disease, heart failure, cardiomyopathy and atrial fibrillation.[204] Other approaches have used EV-mimetics and their bioactive protein cargo from stem cells for cardiac cell repair. Lozano *et al.*[205] integrated quantitative proteomics to understand cell remodelling following treatment to reveal the upregulation of angiogenic proteins (MFGE8, MYH10, VDAC2) in endothelial cells and pro-survival proteins (CNN2, THBS1, IGF2R) in cardiomyocytes, while attenuation of cardiac fibrosis and extracellular matrix remodelling capacity in cardiac fibroblasts (ACTN1, COL1A1/2/4A2/12A1, ITGA1/11, THBS1).[205] This study presents a scalable approach to generating functional EV-mimetics to deliver protein cargo and remodel the cell and signaling landscape for cardiac repair.

A critical challenge of EV-based therapy in the management of cardiovascular diseases is the lack of a robust systemic administration regime to deliver sufficient amounts of EVs to the site of the diseased cells/tissue, followed by the meaningful therapeutic retention of the EVs. Aptamers are ideal candidates for targeted EV therapeutics for cardiovascular diseases due to their high specificity, ease of cellular internalization and rapid tissue penetration and accumulation.[206]

Two groups of researchers have made inroads into the development of aptamers targeting cardiac muscle cells. Catalucci and colleagues were the first to report the use of an internalizing aptamer for the selective delivery of a small therapeutic peptide to cardiac cells.[207] These researchers generated an aptamer-peptide chimera by conjugating the PDGFRβ-targeting Gint4.T aptamer to a small mimetic peptide (MP). The MP has been shown to restore myocardial function in pathological heart conditions associated with defective L-type calcium channel (LTCC) function peptide *via* targeting of the Cavβ2 subunit of the LTCC. When applied to HL-1cardiac muscle cells that contract and retain phenotypic characteristics of the adult cardiomyocyte,[208] the Gint4.T-MP aptamer-peptide chimera was successfully internalized in cells and was able to not only restore LTCC protein levels but also recover LTCC-dependent calcium fluxes in the cardiac cells.[207] Nonetheless, PDGFRβ is expressed in many tissues/organs outside the heart.[209] The development of aptamers with excellent cardio-specificity will advance the development of effective new strategies for the treatment of cardiovascular disease. The heart remains a dauting challenge for developing therapies directed specifically at only one cell type, as cardiomyocytes constitute most of the cell mass but only account for 30% of the total cell number in the heart.[210] It was only until 2023 that cardiac muscle-specific aptamers were developed.[211] To develop targeted therapy for dilated cardiomyopathy, a type of heart muscle disease that causes the heart ventricles to thin and weaken, with very poor five-year survival rates,[212] Phylactou and colleagues isolated a 2′-fluoropyrimidine-modified RNA aptamer, Aptamer-10478.[211] This RNA aptamer has high selectivity towards cardiomyocytes as well as good stability in the serum. Future endeavours in isolating aptamers specific towards cells/tissues underlying cardiovascular pathogenesis will advance the clinical translation of this novel cell-free therapeutics for cardiovascular diseases.

7. Conclusion and perspectives

Oligonucleotide drugs, including aptamers, have gained exceptional scientific and pre-clinical interest, with numerous drugs being approved or used in clinical trials.[213] To date, there are

more than 18 marketed products based on antisense oligonucleotides, aptamers and small interfering RNAs, and many others are in the pipeline for both academia and industry.[214] A major technological trigger for this development has been progress in oligonucleotide chemistry to overcome inherent limitations in the pharmacokinetics of free drugs and reduce the cost of manufacturing. Such strategies focus on enhancing the stability of native RNA and DNA aptamers to minimize degradation by nucleases.[215] Although extensive oligonucleotide libraries of sufficient sequence complexity exist, searching only for sequence space often yields aptamers that are limited in specificity and particularly in affinity, with the field moving towards incorporating chemical and structure space to identify mature functional aptamers.[216] Because the main hurdle for pharmacotherapy is delivery to target tissues, the adoption of delivery technologies, such as nanoparticles and, recently, EVs, is a game changer for many therapeutic indications.

As discussed, EVs as cell-derived lipid-based nanocarriers are remarkable in their biocompatibility and potential for highly specific active targeting through the surface display of endogenous cellular ligands. Moreover, owing in large part to the complex, multimodal biogenesis pathways responsible for their formation and release, EVs contain a diverse molecular cargo that overcomes redundancy in intercellular signalling. Their inherent capacity to transverse cell membranes and transfer such complex bioactive cargo, as well as their ability to be modified through various engineering and modification strategies have attracted significant therapeutic interest.[221,222] With further refinement, molecular bioengineering strategies are providing a new frontier for EV-based therapy, including novel mRNA vaccines, antigen cross-presentation and immunotherapy, organ delivery and repair, and cancer immune surveillance and targeted therapeutics.[50,221] The main challenges for using EVs as delivery systems are (i) reproducible, large–scale production,[217] (ii) effective loading of drugs or reprogramming of the EV cargo directly (through genetic manipulation), and (iii) target selectivity to enhance delivery and retention at the site of the disease. Despite the prolonged circulatory half-life of cargo encapsulated within EVs, most therapeutic EVs tend to accumulate in the liver, with only a small fraction of the injected dose reaching the intended target cells or tissues.[218] Additional breakthroughs are required to improve cell- or tissue-specific delivery. Functional and tissue-targeted modification of EVs from cells used in cell therapy, such as cardiosphere-derived cells (in Phase III clinical trial NCT05126758 (**https://clinicaltrials.gov/ct2/show/ NCT05126758**)) or other stem cells, would further increase efficacy in target tissues.

As aptamers provide numerous biological, chemical, and therapeutic advantages, including their size, selectivity, versatility, and multifunctional programming, their integration into the field of EVs contributes to the development of isolation, detection, and analysis pipelines for therapeutic and diagnostic utility. Aptamers have largely improved the sensitivity in EV detection coupled with some flexible technologies, such as fluorescence signal amplification, energy transfer, nanozymes

catalysis, conversion to ssDNA detection, and sandwich format sensors. Although there are still some crucial technological issues and challenges to be addressed, these center on the stability of aptamers in biological settings *in vivo* and the specificity of aptamer interactions, compounded by the molecular diversity and abundance within and on the surface of EVs. An in-depth analysis of EVs as drug delivery entities may generate valuable insights that can be applied to synthetic particles. However, there are inherent pharmacokinetic challenges with such approaches. The protein corona, an absorption surrounding particles when circulating *in vivo*, makes nanocarriers prone to be recognized by the innate immune system and eliminated by phagocytic cells in the lungs, liver, and spleen.[82] Therefore, merely coating cell-derived EVs, artificial EVs, or hybrid EV systems with aptamers may not adequately fulfill the intended targeting purpose *in vivo*. Innovative approaches, such as modifying the EV membrane and its associated protein corona with active peptides,[219,220] promise to enhance EV targeting and therapeutic efficacy.

The successful translation of aptamer technology into EV-based medical applications depends on the advances in resolving several key limitations of aptamers: (i) the lack of the repeatability of aptamer-target binding due to inherent structural flexibility of oligonucleotides; (ii) the vulnerability of aptamer to its reaction environment in the absence of a standard protocol; (iii) fabricating procedures for many aptamer-based platforms are tedious, making them unfavorable for mass production; and (iv) given the uniqueness of each aptamer, transitioning from one aptamer to another on a particular sensing or isolation platform can be complex and challenging.

There is a growing interest in and convergence between aptamer technology and the EV field. Sequence-specific libraries are overcoming some of the inherent challenges with aptamer targeting and specificity to address fundamental questions in the EV field, namely the origin, form and function of EVs. Recent efforts have focused on identifying novel aptamer targets and stable EV signatures associated with a particular cell, disease or condition. Such technical developments will provide a new understanding of EV biology and application and ultimately translate our rapidly expanding understanding of the diversity of EVs from cells, organs and organisms to novel diagnostics and therapeutics.[221,222]

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgements

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