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REVIEW

Extracellular Vesicles as Emerging Drug Delivery Platforms in Triple-Negative Breast Cancer: A Systematic Review

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Abstract: Breast cancer is the most prevalent malignancy among women, with triple-negative breast cancer (TNBC) being the most aggressive subtype. The lack of standard targeted therapies necessitates reliance on chemotherapy, which often suffers from poor targeting and significant off-target toxicity. This systematic review evaluates the therapeutic potential of drug delivery systems based on extracellular vesicles (EVs) for TNBC using 17 studies from 2020 to 2024 published in Scopus, PubMed, and Web of Science. The review discusses the diverse therapeutic cargos delivered by EVs, including chemotherapeutic agents and nucleic acids, while emphasizing the need for standardized protocols to facilitate EV isolation and characterization. Key findings provide both in vitro and in vivo evidence of the efficacy of EV-based therapies against TNBC cell lines and animal models, alongside insights into engineering strategies that enhance targeting specificity. Despite the promising findings, challenges remain in clinical translation, such as technical limitations and inconsistent reporting. The results underscore the potential of EVs to revolutionize TNBC treatment strategies, paving the way for more effective therapeutic options.

Keywords: exosome, breast cancer, drug delivery system, precision medicine, chemotherapy

Introduction

Breast cancer is the most prevalent cancer in women globally, and triple-negative breast cancer (TNBC) is its most aggressive and metastatic subtype.[1–4] Since targeted receptors are absent, chemotherapy is the primary treatment strategy.[5–9] However, TNBC has high rates of recurrence, poor prognosis, and unfavorable survival outcomes.[10–14] Clinical efficacy of chemotherapeutics is hampered by several limitations, including poor tumor targeting, rapid systemic clearance, and substantial off-target toxicity.[15–21]

Precision medicine has been developed recently and represents a paradigm transformation in oncology to provide more efficient and customized treatment approaches.[22–27] In this context, extracellular vesicles (EVs) have garnered increasing attention as a novel drug delivery platform.[21,28–32] EVs are nanoscale vesicles found in almost all kinds of cells in the body and are important as mediators of intercellular communication through the transport of nucleic acids (DNAs and RNAs), proteins, and lipids.[33–37] Due to their intrinsic biocompatibility, low immunogenicity, natural targeting ability, and adaptability, EVs are excellent candidates for precision medicine.[29,38–40] Increasing evidence supports this potential, demonstrating their ability to selectively deliver therapeutic payloads to target cells, thereby enhancing anti-tumor efficacy while minimizing systemic toxicity and off-target effects.[41–47]

This systematic review examines the therapeutic potential of EV-based drug delivery systems in TNBC, reflecting the growing interest and promising applications of EV-based therapeutics in cancer treatment. Based on an assessment of 17 eligible studies, this review provides: (1) an evaluation of the in vitro and in vivo efficacy of EV-based therapeutic agents in TNBC cell lines and models; (2) an overview of the engineering strategies used to develop EV-based therapeutics,

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with emphasis on targeting specificity and delivery efficiency; (3) a detailed analysis of EV characteristics, including cellular origin, isolation and purification techniques, characterization methods, and storage conditions; (4) a quality assessment of the included studies, focusing on adherence to the MISEV2023 guidelines and in vivo validation; and (5) a translational perspective highlighting clinical challenges and opportunities informed by insights from nanotechnology, pharmacology, and cancer biology to guide future clinical applications. Overall, this review aims to provide a systematic synthesis of the current state of the art and to underscore key directions for advancing EV-based delivery systems as precision therapeutics for TNBC.

Methods

This systematic review was conducted using Scopus, PubMed, and Web of Science (WOS) databases to identify relevant publications from the past five years (2020–2024). Access to these databases was provided by the National University of Malaysia (UKM). The search query used was "extracellular vesicles OR exosomes" AND "drug delivery systems" AND "triple-negative breast cancer", formulated based on terms obtained from the Medical Subject Headings (MeSH) database. Common terms identified from several relevant published studies were also incorporated into the search strategy. All search records, including titles, keywords, and abstracts, were downloaded and imported into EndNote. Duplicate records were removed before further screening. The initial screening excluded review articles, followed by an evaluation of titles, abstracts, and keywords for relevance to the topic. Subsequently, the full-text articles were retrieved and assessed for eligibility. The final screening was conducted based on the predefined inclusion and exclusion criteria. Inclusion criteria comprised articles reporting the use of extracellular vesicles (EVs) or exosomes (EXOs) as drug delivery systems for TNBC in in vitro, animal, or human studies. Exclusion criteria included review articles, conference abstracts, non–peer-reviewed publications, and studies not published in English. Subsequently, the in vivo studies were assessed for risk of bias using SYRCLE's Risk of Bias (RoB) tool for animal studies. The review protocol was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines and has been registered in the International Prospective Register of Systematic Reviews (PROSPERO) under ID: CRD420250637660.

A total of 151 records were retrieved from three databases: Scopus (62), PubMed (38), and WOS (51). After removing 45 duplicates and 41 review articles, 65 records remained for further analysis. Subsequent screening based on titles, abstracts, and keywords excluded 38 records, leaving 27 articles for full-text assessment. Of these, 10 articles were excluded according to the predefined inclusion and exclusion criteria, resulting in a final set of 17 articles for data extraction and analysis. Among these, 6 studies reported only in vitro outcomes, 11 studies included both in vitro and in vivo outcomes, and none reported clinical outcomes. Article selection and data extraction were independently performed by two reviewers, with the corresponding author consulted to resolve discrepancies and reach consensus, thereby minimizing selection bias. The summary of the article selection protocol is reported in Figure 1.

Results and Discussion

EV-Based Therapeutic Delivery Systems for TNBC: in vitro and in vivo Evidence

This section summarizes the experimental outcomes of EV-based delivery systems in TNBC, focusing on the antitumor effects observed in in vitro cell culture and in vivo animal model studies (Table 1). It provides insight into the therapeutic efficacy and translational potential of EV-based platforms.

All 17 studies included in this review conducted in vitro experiments to evaluate the antitumor efficacy of EV-based delivery systems against various TNBC cell lines. As summarized in Table 2, the delivery platform demonstrated effectiveness against a broad spectrum of TNBC cell lines, including human cell lines (MDA-MB-231, MDA-MB-468, HCC1806, HCC1937, BT-20) and the murine 4T1 line. Among these, the most utilized cell lines were MDA-MB-231 (11 studies)[44,49–51,54,56,58–60,62,63] and 4T1 (6 studies).[48,52,53,55,57,61]

In vitro assessment of cellular uptake and cytotoxicity represents a critical initial step in evaluating the therapeutic potential of EV-mediated delivery systems. To assess uptake and targeting efficiency, 13 studies[44,48–52,54,55,57,59,61–63] employed fluorescent labeling combined with confocal microscopy or flow cytometry to monitor internalization of EV-

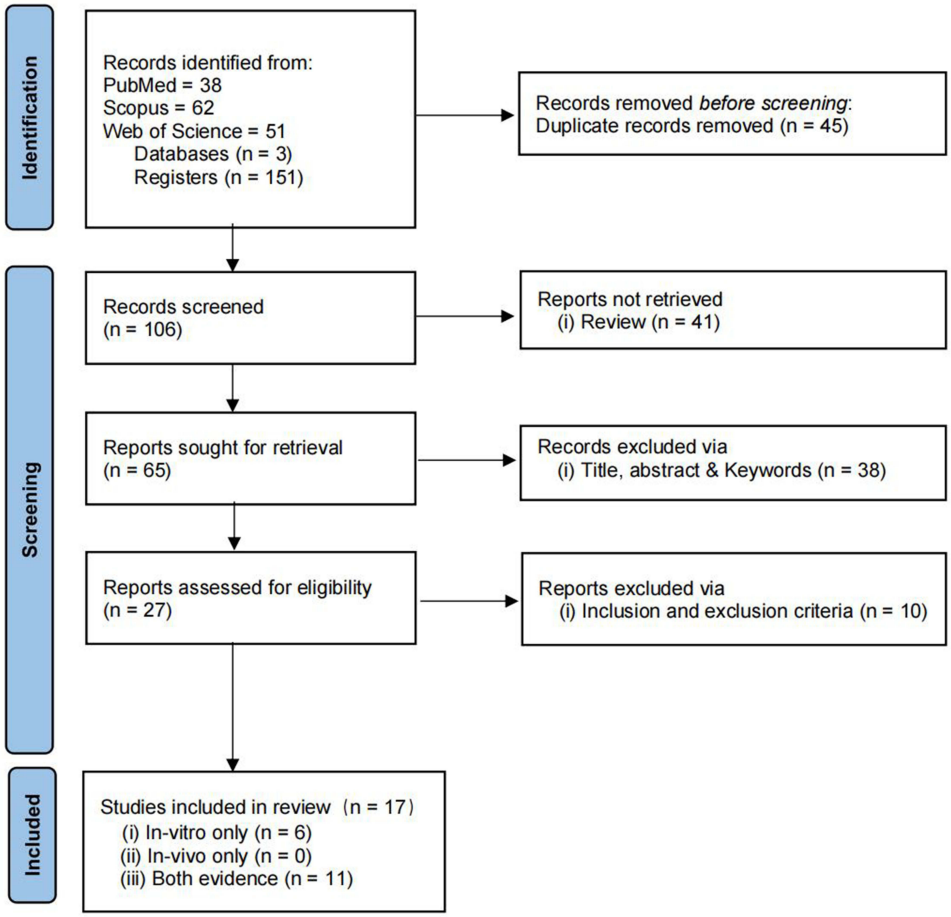

Figure 1 PRISMA flow chart summarizing the study selection process.

loaded cargo by TNBC cells. Notably, Pullan et al[59] and Hashemi et al[49] established three-dimensional (3D) cell culture models in vitro to further confirm the cellular uptake of EV-loaded drugs under physiologically relevant conditions. Cytotoxicity assays were the most common method used to quantify anticancer effects, including MTT[50,51,54,56,57,60,61] and Cell Counting Kit-8 (CCK-8).[48,52,53,55,62,63] Other studies used cell viability assays such as CellTiter-Blue[52] and CellTiter-Glo.[44] Furthermore, apoptosis induction in the cancer cells treated with EV-loaded drugs was confirmed using Annexin V/PI staining or the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.[50,51,56,60] Collectively, these in vitro studies showed efficient cellular uptake, selective cytotoxicity toward TNBC cells, and

Table 1 Overview of EV-Based Drug Delivery in TNBC: In Vitro and In Vivo Outcomes

Study	In vitro	In vivo
Guo et al, 2021[48]	OMVs co-deliver PTX/siRNA into TNBC cells, reprogramming TAMs and enhancing tumor phagocytosis via pH-triggered release.	siRNA@M-/PTX-CA-OMVs exhibited enhanced TAM-targeted accumulation and pH-responsive drug release, leading to significant TNBC tumor suppression via immune activation.
Hashemi et al, 2023[49]	SFB-NK-Exos selectively kill both 2D and 3D TNBC culture cells via enhanced apoptosis with reduced off-target toxicity.	—
Shojaei et al, 2021[50]	miR-381-loaded ADMSC exosomes suppress TNBC progression by inhibiting Wnt/EMT pathways and enhancing apoptosis, while reducing migration/invasion.	—

(*Continued*)

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Table 1 (Continued).

Study	In vitro	In vivo
Li et al, 2020[51]	MEP-D exhibits pH-responsive drug release, and enhanced cellular uptake via c-Met targeting, and superior apoptosis induction in TNBC cells compared to PL-D/EP-D.	MEP-D achieved enhanced tumor targeting and retention via c-Met-specific delivery, resulting in superior TNBC tumor growth inhibition with favorable biosafety.
Bose et al, 2022[52]	uPAR-targeted 4T1-eEV-PNCs enhanced antimiRNA-21/10b delivery and synergized with low-dose doxorubicin, enhancing TNBC cell uptake and cytotoxicity.	uPAR-targeted eEV-PNCs synergized with low-dose doxorubicin, enabling tumor-specific accumulation and 40% complete regression in 4T1 models with low toxicity.
Y. Ding et al, 2023[53]	Ce6-GW4869/sEVs effectively induce immunogenic cell death in TNBC cells via PDT-mediated CRT exposure, HMGB1/ATP release, and tumor vaccination effects.	Ce6-GW4869/sEVs enhance TNBC targeting and antitumor immunity via PDT-induced ICD and TME modulation.
M. J. Haney et al, 2019[54]	EVs enhance drug uptake in TNBC cells via adhesion proteins, outperforming conventional liposomes.	EV-drug systems show stronger antitumor effects than commercial drugs in TNBC models.
K. Zhang et al, 2021[55]	ABAs exhibits homologous targeting in 4T1 cells, and macrophage evasion via CD47, with dose-dependent cytotoxicity through Twist silencing.	ABAs demonstrates 2.7-fold enhanced tumor accumulation over liposomes with prolonged circulation and superior anti-metastatic efficacy in TNBC models.
D. Uslu et al, 2024[56]	PLT-Exo-Dox exhibits pH-responsive drug release, enhanced tumor-specific cytotoxicity, and pro-apoptotic effects via Bax/Bcl-2 modulation in MDA-MB-231 cell line.	—
M. Basak et al, 2024[57]	Exo ($PAN_{34a+DTX}$) outperforms individual components (PAN/DTX/Exo) in transfection, cytotoxicity and Bcl-2 suppression in 4T1 cells.	—
N. Erwin et al, 2024[58]	μDES-generated PROTAC EVs demonstrated superior HDAC3/8 degradation compared to conventional electroporation, with rapid and sustained effects.	μDES-PROTAC EVs safely suppressed TNBC tumors via HDAC3/8 degradation without cross-species activity or systemic toxicity.
J. Pullan et al, 2022[59]	iDHRX exhibited enhanced TNBC cell uptake and cytotoxicity under both normoxic and hypoxic conditions, with efficient 3D penetration.	—
R. Sarkar et al, 2024[60]	e-DDMSNPs exhibited superior cellular uptake and tumor penetration in TNBC cells and 3D spheroids compared to free drugs or plain nanoparticles.	e-DDMSNPs outperformed DOX in tumor suppression and metastasis prevention with reduced toxicity.
Y. Si et al, 2022[44]	EGFR/CD47 mAb-EVs showed high binding affinity to TNBC cells and were efficiently internalized, supporting their effective drug delivery capability.	Dual-targeted EGFR/CD47 mAb-EVs effectively inhibited TNBC tumor growth in xenograft and PDX models without significant off-target toxicity.
M. C. Sun et al, 2023[61]	T-DOX demonstrated greater TNBC cell uptake and cytotoxicity than Doxil, and upregulated PD-L1 and immune-activating markers/signals.	T-DOX followed by S-PDNG achieved superior tumor suppression by boosting immune attack (more CD8+ T-cells, fewer Tregs) with no side effects.
Y. W. Wu et al, 2023[62]	CFDASE-labeled PEVs were more efficiently internalized by breast cancer cells (MDA-MB-231 and MCF7/ADR) than normal fibroblasts (NIH/3T3) via clathrin-mediated endocytosis, and DOX-loaded PEVs exhibited enhanced cytotoxicity in cancer cells while showing reduced toxicity in normal cells compared to free DOX and liposomal DOX.	—

(*Continued*)

Table 1 (Continued).

Study	In vitro	In vivo
Z. Zhang et al, 2024[63]	cRGD-Exos/miR-588 efficiently targeted MDA-MB-231 cells, suppressed TGF-β/CCL5, and induced selective cytotoxicity against TNBC cells while sparing normal cells.	Systemically administered cRGD-Exos/miR-588 significantly inhibited TNBC tumor growth, reprogrammed the TME via M2-to-M1 macrophage polarization, enhanced NK cell cytotoxicity, and showed high tumor-targeting efficiency with minimal systemic toxicity.

Note: Symbol: "—" indicates data not available or not investigated.

Abbreviations: ABAs, apoptotic body analogues; Bax, pro-apoptotic gene; Bcl-2, anti-apoptotic gene; CA, pH-sensitive linker cis-aconitic anhydride; CCL5, C-C chemokine ligand 5; Ce6, GW4869/sEVs-immunomodulatory photosensitive nanovesicle; CFDASE, 5-(and-6)-carboxy-fluorescein diacetate: succinimidyl ester; CRT, calreticulin; cRGD, Exos/miR-588-cyclic RGD-modified exosomes loaded with miR-588; DIM, dietary indole 3,3′-diindolylmethane; DOX, doxorubicin; DTX, docetaxel; e-DDMSNPs, DIM and DOX-loaded mesoporous silica nanoparticles encapsulated in exosomes; EGFR/CD47 mAb, EVs-exosomes modified with epidermal growth factor receptor and CD47 monoclonal antibodies; EP, D-macrophage exosome coated nanoparticle; Exo, exosomes; HDAC 3/8, histone deacetylases 3/8; HMGB1, high-mobility group box 1; iDHRX, tumor-penetrating (i); HR, hypoxia-responsive; D, DOX, chemotherapeutic; X, exosome; ICD, immunogenic cell death; MEP, D-the c-Met-targeting nanoparticle; μDES, microfluidic droplet-based electroporation; OMVs, outer membrane vesicles; PAN, pH responsive polyamine phosphate nanocarrier; PAN34a+DTX, docetaxel and tumor suppressor miR-34a; PD-L1, programmed death-ligand 1; PDX, patient-derived xenograft models; PEVs, platelet extracellular vesicles; PL-D, DOX-loaded poly nanoparticles; PLT-Exo-Dox, DOX-loaded platelet exosomes; PNCs, polymeric nanocarriers; PROTACs, proteolysis targeting chimeras; PTX, paclitaxel; SFB-NK-Exos, sorafenib-loaded NK cell-derived exosomes; sEVs, small extracellular vesicles; siRNA@M-/PTX-CA-OMVs-Redd1, siRNA and PTX-coloaded OMV hybrid nanoparticles; S-PDNGs, TME-responsive disulfide-linked PD1-cross-anchored TDEVs nanogels; T-DOX, Tumor-derived extracellular vesicles PD-L1^{KO}-fusogenic DOX liposomes; TAMs, tumor-associated macrophages; TDEVs, tumor-derived extracellular vesicles; TGF, β-transforming growth factor-beta; TME, tumor microenvironment; uPAR-targeted 4T1-eEV-PNCs, containing PNCs loaded with therapeutic anti-miRNAs and coated with uPA-engineered extracellular vesicles.

apoptosis induction. However, the lack of systemic biological factors limits the translational relevance of in vitro models. Therefore, further investigations involving in vivo models and biodistribution studies are essential to validate the efficacy and specificity of EV-based delivery systems within the complexities of living organisms.

Biodistribution assessment is a crucial parameter for validating the in vivo tumor targeting efficacy of EV-based drug delivery systems. Among the 17 studies included, ten used various strategies to evaluate the biodistribution of the delivery platform in animals or vital organs.[44,48,51–55,58,61,63] Seven of these employed both in vivo and ex vivo fluorescence or bioluminescence imaging, while Zhang et al[55] used ex vivo imaging only, and Sun et al[61] and Zhang et al[63] performed in vivo imaging without ex vivo validation. Intravenous injection (i.v.) was the most common administration route (9 studies), with Erwin et al[58] using intraperitoneal injection (i.p.). Notably, Haney et al[54] compared biodistribution outcomes across i.v., i.p., and intratumoral (i.t.) routes. A range of fluorescent dyes was used for imaging, with DiR being the most frequently used,[58,61,63] followed by Cy5[51,55] and BODIPY,[48] Ce6,[53] DiD,[54] Cy7,[44] and ICG.[52] These dyes are generally classified into two groups: fluorescent imaging dyes, such as BODIPY, Cy5, Cy7, DiD, DiR, and ICG, and photosensitizers used for photodynamic therapy, such as Ce6. Although imaging techniques, administration routes, and dye selections varied, all ten studies consistently showed that EV-based delivery systems effectively targeted TNBC cells. Off-target distribution was minimal in each case.

A total of 11 studies conducted in vivo experiments to examine the efficacy of EV-based drug delivery against TNBC. Details of the in vivo studies, including animal models, gender, group, sample size, administration routes, and injection frequencies, are provided in Table 2. Diverse mouse models, such as BALB/c mice,[48,52–54,60,61] BALB/c nude mice,[55,63] BALB/cJ mice,[44] nude mice,[51,54] and NOD SCID gamma (NSG) mice,[44,58] were utilized to establish tumor-bearing models in these studies. Among these in vivo studies, nine studies delivered therapeutic agents to animal models via i.v. tail vein injections.[44,48,51–55,61,63] Alternative routes of administration, including i.t.[60] and i.p.[58] were also utilized to deliver cargoes entrapped in EVs to mouse models. Compared to i.v. and i.p., i.t. appears to require fewer injections. Sarkar et al[60] administered therapeutic agents intratumorally to models with only one dose, whereas other studies employed multiple injections. All 11 in vivo studies demonstrated impressive antitumor effects of the EV-based drug delivery platform in TNBC animal models, shown by decreased tumor size and weight, as well as reduced side effects indicated by less body weight loss and higher survival rates. Across all 11 studies, one evaluated the antitumor effects only by tumor size.[54] One study measured the expression of TNBC tumor-specific proteins by Western blot to evaluate the targeting antitumor effects of the EV-based therapy.[58] Nine employed histopathology to further validate qualitatively and quantitatively the in vivo anticancer effects.[44,48,51–53,55,60,61,63] All nine studies used Hematoxylin & Eosin (H&E) staining to evaluate the anti-tumor effect,

Table 2 Summary of in Vivo Evaluation of EV-Based Delivery Systems for TNBC

Study	EV-based therapeutic agent	Generating strategy	Animal model				Administration Strategy					Biodistribution					
			Tested cell	Animal	Gender	Sample size	Group	Dosage	Route	Frequency	Euthanasia	Type	Dye	Route	Dosage/ Frequency	Detect type	Timepoints
Guo et al, 2021[48]	siRNA@M-/PTX-CA-OMVs	Electroporation for Redd1 siRNA, incubation for DSPE-PEG-CA-PTX and CRISPR-mediated gene knockout	4T1 cells	BALB/c mice	Female	7 groups (n=10)	Control	—	i.v.	Every 3 days for 2 weeks	Day 20	In vivo	BODIPY	i.v.	—	IVIS	1, 2, 4, 8, 12, 24 hours
							OMVs										
							siRNA@OMVs										
							siRNA@M-OMVs										
							siRNA@PTX-CA-OMVs					Ex vivo	Cy3 labeled CD34 +DAPI	i.v.	—	LSCM	24 hours
							siRNA@M-/PTX-OMVs										
							siRNA@M-/PTX-CA-OMVs										
Li et al, 2020[51]	MEP-D	Co-extrusion for PLGA nanoparticles, surface engineering for the c-Met binding peptides	MDA-MB-231 cells	Nude mice	—	5 groups	PSB	0.01M	i.v.	Every 3 days	—	In vivo	Cy5.5	i.v.	1 dose, Day 0	Fluorescence imaging	4, 8, 12, 24 hours
							DOX	0.01M									
							PL-D	0.01M									
							EP-D	0.01M				Ex vivo	Cy5.5	i.v.	1 dose, Day 0	Fluorescence imaging	24 hours
							MEP-D	0.01M									
Bose et al, 2022[52]	eEV-PNCs	Transfection for uPA/Sc-uPA peptide, extrusion for PLGA anti-miR-21/anti-miR-10b	4T1 cells	BALB/c mice	—	4 groups (n=10)	Saline	—	—	—	—	In vivo	ICG	i.v.	3 doses, Day 0, 6, 12	NIRF	2, 7, 15 days
							DOX	—								PAI	16 days
							Sc-uPA nano cocktail	—				Ex vivo	ICG	i.v.	3 doses, Day 0, 6, 12	NIRF	17 days
							uPA nano cocktail	—									
Y. Ding et al, 2023[53]	Ce6-GW4869/ sEVs	Electroporation	4T1 cells	BALB/c mice	Female	5 groups	Saline	—	i.v.	Every other day (3 doses)	Day 14	In vivo	—	i.v.	1 dose, Day 0	Fluorescence imaging	1, 2, 4, 8, 12, 24, 48, 72 hours
							sEVs	—									
							Ce6/sEVs	—									
							GW4869/sEVs	—				Ex vivo	—	i.v.	1 dose, Day 0	Fluorescence imaging	72 hours
							Ce6-GW4869 /sEVs	—									

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M.J. Haney et al, 2019[54]	EV-PTX EV-DOX	Sonication	MDA-MB-231 cells	Nude mice	—	4 groups (n = 7)	Saline	—	i.v.	Day 1, 4, 7, 9	First endpoint in saline group	Ex vivo	DID	i.v. i.t. i.p.	1 dose, Day 0	Confocal fluorescence microscopy	4 hours
							Taxol	—									
							EVs	—									
							EV-PTX	0.5 mg/kg									
			8FlmC-FLuc-T11 cells	BALB/C mice	—	5 groups (n = 7)	Saline	—	i.v.	Day 1, 4, 7, 9	First endpoint in saline group						
							DOX/saline	2.5 mg/kg									
							Doxil	—									
							EV-DOX	—									
K. Zhang et al, 2021[55]	ABAs	Surface engineering for PFA/DTT, electroporation for toxic protein saporin and anti-Twist siRNA	4T1 cells	BALB/c nude mice	Female	4 groups (n =5)	Saline	—	i.v.	Every 3 days (3 doses)	Day 21	Ex vivo	Cy5	i.v.	1 dose, Day 0	Fluorescence imaging	24 hours
							Saporin + anti-Twist siRNA	—									
							Liposomes	310 ug/kg saporin +1 mg/kg anti-Twist siRNA									
							ABAs	310 ug/kg saporin +1 mg/kg anti-Twist siRNA									
N. Erwin et al, 2024[58]	PROTAC-EVs	Electroporation (μDES)	MDA-MB-231 cells	NSG mice	—	—	PBS	0.1mL	i.p.	3 times per week for 5 weeks	Day 35	In vivo	DiR	i.p.	1 dose, Day 0	IVIS	0, 3, 6, 12, 24, 48 hours
							PROTAC	100nM									
							EVs	—				Ex vivo	DiR	i.p.	1 dose, Day 0	IVIS	48 hours
							PROTAC EVs	$1.4x10^{10}$ EVs/mL									
R. Sarkar et al, 2024[60]	e-DDMSNPs	Incubation	4T1 cells	BALB/c mice	Female	5 groups (n=12)	Control	5 mg/kg	i.t.	Once	Day 21	In vivo	—	—	—	IVIS	—
							DOX	—									
							e-DDMSNPs	—									

(*Continued*)

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Table 2 (Continued).

Study	EV-based therapeutic agent	Generating strategy	Animal model				Administration Strategy					Biodistribution					
			Tested cell	Animal	Gender	Sample size	Group	Dosage	Route	Frequency	Euthanasia	Type	Dye	Route	Dosage/ Frequency	Detect type	Timepoints
Y. Si et al, 2022[44]	mAb-EV-Ver-A	Surface engineering for anti-human EGFR and CD47 mAbs, incubation for Ver-A	4T1 cells	BALB/cJ mice	Female	8 groups (n =5–6)	PBS	—	i.v.	Day 8, 10, 13, 15	Tumor volume reached >1000 mm^3 in the PBS group	In vivo-	Cy7	i.v.	1 dose, Day 0	IVIS	24 hours
							EV	—									
							EGFR-EV-Ver-A	0.5 mg/kg									
							CD47-EV-Ver-A	0.5 mg/kg									
							EGFR/CD47-EV-Ver-A	0.5mg/kg									
								1.5mg/kg									
								2 mg/kg									
								2.5mg/kg									
				NSG mice	female	3 groups (n=5)	PBS	—	i.v.	Day 0, 4, 9, 15, 21, 26	Tumor volume reached >1000 mm^3 in the PBS group	Ex vivo	Cy7	i.v.	1 dose, Day 0	IVIS	24 hours
							mAb-EV	—									
							mAb-EV-Ver-A	4mg/kg									

M. C. Sun et al, 2023[61]	T-DOX	CRISPR/Cas9 gene-editing technique in 4T1 Pd-l1^{KO}	4T1 cells	BALB/c mice	Female	5 groups (n = 5)	PBS	—	i.v.	Once	Day 23	In vivo	DiR	i.v.	1 dose, Day 0	IVIS	4, 8, 12, 24, 48 hours
							CIS	10 μM									
							DOX Sol	—									
							Doxil	—									
							T-DOX	80 μM(DOX)									
				BALB/c mice	Female	3 groups (n=5)	PBS	—	i.v.	Day 1, 4, 7, 9	Day 15						
							PD1 Sol	—									
							S-PDNGs	50 μg (PD1)									
				BALB/c mice	Female	5 groups (n=3)	PBS	—	i.v.	—	Day 24						
							1TP	—		Day 1, 2							
							1PT	—		Day 1, 2							
							7TP	—		Day 1, 8							
							7PT	—		Day 1, 8							
				BALB/c mice	Female	4 groups (n=5)	PBS	—	i.v.	Day 0, 2, 4, 6, 8	Day 15						
							Doxil	—		Day 1, 5, 8, 10							
							T-DOX	—		Day 1, 5, 8, 10							
							T-DOX +S-PDNGs	5 mg kg−1 DOX, 50 μg PD1/mouse		Day 1, 5, 8, 10 (T-DOX) Day 2, 6, 9, 11 (S-PDNG)							
Z. Zhang et al, 2024[63]	cRGD-Exos /miR-588	Electroporation for miR-588 mimic, surface engineering for DSPE-PEG2000-cRGD	MDA-MB-231 cells	BALB/c nude mice	Female	3 groups	Saline	200 μL	i.v.	Day 0, 3, 6, 9, 12	Day 14	In vivo	DiR	i.v.	1 dose, Day 0	Fluorescence imaging	1, 4, 8, 12, 24 hours
							miR-588	5 nmol									
							cRGD-Exos/miR-588	1×10^{10} particles/ each									

Note: Symbol: "—" indicates data not applicable or not investigated.

Abbreviations: 1TP/1PT or 7TP/7PT, T-DOX and S-PDNGs consecutively, but in the opposite order, with a 1- or 7-day interval; 4T1 Pd-l1^{KO}, murine breast cancer cell (4T1) line with Pd-l1 knockout; ABAs, apoptotic body analogues; BODIPY, boron dipyrromethene; Ce6-GW4869/sEVs, immunomodulatory photosensitive small extracellular vesicles; c-Met, mesenchymal epithelial transition; cRGD-Exos/miR-588, cyclic RGD-modified exosomes loaded with miR-588; CIS, cis-platinum; DAPI, 4′,6-diamidino-2-phenylindole; DIM, dietary indole 3,3′-diindolylmethane; DiR, 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide; DID, 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindodicarbocyanine, Perchlorate; DOX, doxorubicin; DOX-Sol, doxorubicin solution; DSPE-PEG$_{2000}$, 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-[(polyethylene glycol)$_{2000}$]; DTT, dithiothreitol; e-DDMSNPs, DIM and DOX-loaded mesoporous silica nanoparticles encapsulated in exosomes; EGFR/CD47 mAb-EVs, exosomes modified with epidermal growth factor receptor and CD47 monoclonal antibodies; EP-D, empty macrophage exosome coated PLGA nanoparticles; EVs, extracellular vesicles; eEVs, engineered extracellular vesicles; ICG, indocyanine green; i.p., intraperitoneally; i.t., intratumorally; i.v., intravenously; IVIS, in vivo imaging system; LSCM, laser scanning confocal microscopy; MEP-D, c-Met-targeted exosomal membrane coated PLGA nanoparticles; mAb, monoclonal antibody; NIRF, near-infrared fluorescence imaging; NSG, NOD scid gamma mice; OMVs, outer membrane vesicles; PAI, photoacoustic imaging; PBS, phosphate-buffered saline; PD1 Sol, PD1 inhibitor solution; PFA, paraformaldehyde; PL-D, DOX-loaded PLGA nanoparticles; PLGA, poly lactic-co-glycolic acid; PNCs, polymeric nanocarriers; PROTACs, proteolysis targeting chimeras; PTX, paclitaxel; S-PDNGs, tumor microenvironment-responsive PD1-cross-anchored tumor-derived extracellular vesicle nanogels; Sc-uPA, scrambled urokinase plasminogen activator; siRNA@M-/PTX-CA-OMVs, Redd1-siRNA and PTX-coloaded OMV hybrid nanoparticles; T-DOX, tumor-derived extracellular vesicles Pd-l1KO-fusogenic doxorubicin liposomes; uPA, urokinase plasminogen activator; Ver-A, verrucarin A.

safety, and anti-metastatic effect of EV-based therapeutics by visually observing changes in tumor tissue size, apoptosis of main organs in the body, and structural changes in common metastatic organs.[44,48,51–53,55,60,61,63] Si et al[45] and Zhang et al[63] also utilized immunohistochemistry (IHC) to demonstrate the visualization and quantification of specific proteins associated with antitumor effects. Furthermore, some studies utilized immunofluorescent staining[60] and flow cytometry[61,63] to examine immune-related aspects of EV-based agents.

The integration of advanced imaging techniques with quantitative analysis and histopathological assessment revealed both the therapeutic potential and safety profile of EV-based drug delivery systems, establishing a strong basis for clinical translation.

Therapeutic Cargos and Engineering Strategies in EV-Based Delivery Systems

This section provides a comprehensive overview of EV based delivery systems in TNBC, highlighting the diverse therapeutic cargos, including chemotherapeutic drugs, nucleic acid therapeutics and bioactive compounds, delivered via EVs, as well as the engineering strategies employed for cargo loading and EV modification, as reported across the 17 studies (Table 2). Notably, nine studies reported improved anti-tumor efficacy with EV-encapsulated chemotherapeutics, while six focused on nucleic acid delivery and another six on bioactive molecules. Chemotherapeutics were most common, with doxorubicin (DOX),[51,54,56,59–62] paclitaxel (PTX),[48,54] docetaxel (DTX),[57] and sorafenib (SFB)[49] frequently featured. The nucleic acid therapeutics included siRNAs (eg, Redd1-siRNA, anti-Twist siRNA), miRNA mimics (miR-34a, miR-381-3p, miR-588), and anti-miRNAs (anti-miR-21, anti-miR-10b). Another group of studies explored EVs carrying bioactive molecules such as Ce6/GW4869 photosensitizers, saponin, DIM, Ver-A, YX968 PROTACs, and polymeric nanoparticles. This diversity of loaded cargos reflects the adaptability and potential of EV-based systems in TNBC treatment.

Regarding cargo loading techniques, electroporation was the most commonly employed method (used in 9 studies).[48–50,53,55,56,58,59,63] Notably, Erwin et al[58] introduced a continuous-flow microfluidic droplet-based electroporation (μDES) approach, which achieved superior loading efficiency and long-term stability compared to conventional electroporation. Alternative loading techniques used in the reviewed studies included incubation,[44,48,57,60,62] co-extrusion[51,52,61] as well as sonication.[54]

To further enhance delivery specificity and efficacy, EV modification strategies were employed in eight studies. These included both genetic engineering of donor cells[48,52,55,61] and surface functionalization techniques, such as lipid[59] or polymer insertion.[63] Si et al[44] developed dual-targeted mAb-EVs (EGFR/CD47), which showed selective uptake by both human and murine TNBC cells and improved therapeutic efficacy. Li et al[51] constructed PLGA nanoparticle-coated EVs, enhancing delivery precision and stability.

Each generating approach presents distinct advantages and limitations. Therefore, continued innovation and comparative evaluation of these strategies are essential to optimize the efficacy, specificity, and translational potential of EV-based drug delivery systems for TNBC.

Characteristics of the Included Study

A comprehensive understanding of both the methodological and biological characteristics of EVs is critical for evaluating their therapeutic application in TNBC, as demonstrated by the detailed examination of the 17 studies included in this systematic review. These studies were analyzed with respect to EV origin, isolation and purification techniques, characterization approaches, and storage conditions, parameters that play a pivotal role in determining the quality, consistency, and translational potential of EV-based drug delivery systems. The following section summarizes the key features and methodological differences among the included studies.

A wide range of EV sources identified across these 17 included studies reflects the innovative and diverse strategies being adopted in the field of TNBC drug delivery. All the studies used mammalian-derived EVs except Guo et al[48] that utilized outer membrane vesicles (OMVs) derived from engineered E. coli (BL21) bacteria. Among the studies used mammalian-derived EVs, six studies[44,52,55,58,60,61] employed tumor cell-derived EVs, four from TNBC cells (including murine 4T1[52,55,61] and human MDA-MB-231 lines[58]) and two from other malignant tumor types.[44,60] Immune cell-derived EVs were reported in four studies, ie, human NK cells,[49] murine RAW 264.7 macrophages,[54,57] and

macrophage[51] (origin not specified). Three studies utilized stem cell-derived EVs, specifically from human adipose-derived stem cells (hADSCs)[50] and mesenchymal stem cells (hMSCs),[63] and murine bone marrow-derived stem cells (mBMSCs),[53] showcasing their regenerative and targeting potential. The remaining three studies explored less conventional sources, ie, human platelets[56,62] and raw bovine milk.[59]

EVs derived from different cell types possess distinct physicochemical and biological properties, which can significantly influence their targeting behavior and the responses of recipient cells.[64] Numerous studies have demonstrated that tumor cell-derived EVs exhibit strong homologous targeting capability, which may be attributed to the specific membrane proteins inherited from parental cancer cells that selectively facilitate drug uptake by homologous tumor cells.[52,55,58,61] For instance, Zhang et al[55] conjugated the toxic protein saporin and anti-twist siRNA to 4T1 cell-derived exosomes (ABAs) and co-incubated them with RAW264.7 macrophages to investigate their anti-phagocytic behavior. The results showed that ABAs inherited the anti-phagocytic properties from parental tumor cells and effectively evaded macrophage uptake. Furthermore, in vivo studies confirmed that autologous tumor-derived EVs suppressed lung metastasis by enhancing the expression of Twist and promoting epithelial-mesenchymal transition (EMT). Similarly, Erwin et al[58] loaded proteolysis targeting chimera (PROTAC) molecules into MDA-MB-231-derived EVs, which promoted the degradation of histone deacetylases HDAC3/8, leading to activation of tumor suppressor genes and significant antitumor effects. In another study, Sun et al[61] utilized 4T1 cell-derived EVs (TDEVs) to enhance the immunogenic cell death (ICD) effect of doxorubicin (DOX). The treatment increased HMGB1 release and calreticulin (CRT) exposure on the cell surface, thereby enhancing the immunogenicity of 4T1 tumor cells.

EVs derived from immune cells can effectively enhance immunotherapy efficacy while minimizing systemic toxicity.[65] These vesicles also mimic immune cell functions due to the inherent tumor-targeting abilities of their surface proteins. For example, Basak et al[57] designed macrophage-derived exosomes co-delivering docetaxel (DTX) and miR-34a (PAN34a+DTX) and the exosomes were found to inhibit 4T1 tumor growth and promoted apoptosis by upregulating inflammatory cytokines (TNF-α and IFN-γ) and downregulating the anti-apoptotic gene BCL-2. Li et al[51] further modified macrophage-derived exosomes with a c-Met-targeting peptide (MEP-D) for TNBC therapy. In vitro, MEP-D exhibited the highest cellular uptake and apoptotic induction among tested groups. In vivo TUNEL staining confirmed extensive tumor cell apoptosis in the MEP-D-treated group, consistent with in vitro findings. Natural killer (NK) cell-derived EVs have also shown promise. Hashemi et al[49] reported that SFB-loaded NK-EVs demonstrated high tumor selectivity in both 2D and 3D cultures, significantly downregulated VEGF-A expression, and upregulated p53 and caspase-3, thereby enhancing tumor cell apoptosis.

Stem cells are considered ideal sources of EVs because of their self-renewal and regenerative properties.[66] Stem cell-derived EVs have been widely explored as therapeutic delivery vehicles targeting TNBC cells.[50,53,63] Shojaei et al[50] employed adipose-derived MSCs (ADMSC) exosomes to deliver a miR-381 mimic to MDA-MB-231 cells, resulting in increased apoptosis and inhibited proliferation, migration, and invasion. Zhang et al[63] successfully constructed MSC-derived exosomes loaded with miR-588 and modified with cRGD peptides (cRGD-Exos/miR-588). The engineered vesicles exhibited potent TNBC tumor targeting and strong antitumor efficacy by remodeling the tumor microenvironment (TME), reprogramming tumor-associated macrophages (TAMs), and reducing immunosuppression. Interestingly, Ding et al[53] encapsulated the photosensitizer Ce6 and GW4869 into bone marrow MSC-derived small EVs to generate immunomodulatory photosensitized nanovesicles. These constructs improved the tumor immune microenvironment and demonstrated strong tumor-targeting capability both in vitro and in vivo.

EVs derived from platelets also display considerable potential in tumor-targeted drug delivery.[67] Uslu et al[56] and Wu et al[62] demonstrated that doxorubicin-loaded platelet-derived EVs (DOX-PEVs) effectively targeted and enhanced the cytotoxicity of DOX toward MDA-MB-231 breast cancer cells. Moreover, Wu et al[62] showed that PEVs and DOX-PEVs could be sterilized by filtration and stored frozen without procoagulant risk or alteration of surface markers, supporting their suitability for clinical translation.

Notably, Guo et al[48] developed a dual-drug delivery system based on Gram-negative bacteria-derived outer membrane vesicles (siRNA@M-/PTX-CA-OMVs) co-loading paclitaxel (PTX) and Regulated in Development and DNA Damage Response 1 (Redd1)-siRNA. This system effectively repolarized TAMs, remodeled the TME, and activated antitumor immunity to suppress TNBC progression in vitro and in vivo.

Collectively, these findings underscore that EVs from various sources, ie, tumor cells, immune cells, stem cells, platelets, or bacteria, exhibit unique immunomodulatory properties and targeting behaviors. EV-based delivery systems represent a promising and versatile platform for cancer immunotherapy, providing controlled regulation of immune responses and enhanced therapeutic selectivity. Furthermore, emerging studies have revealed that EVs can also serve as carriers of radiosensitizers to improve the precision and controllability of radiotherapy.[68–71] A thorough and ongoing understanding of the biological characteristics and therapeutic potential of EVs from different origins is essential for advancing their use in TNBC treatment and facilitating clinical translation.

The isolation and purification methods of EVs varied notably. As summarized in Table 3 and visualized in Figure 2, differential centrifugation (DC) is the most commonly employed technique, with 12 studies[44,48,51–54,56–59,62,63] utilizing ultracentrifugation (UC) for EV isolation, followed by commercial isolation kits[49,50,60] and extrusion,[55,61,62] each reported in 3 studies. Additionally, another physical disruption techniques, ie, freeze–thaw cycles, were reported in 1 study.[62] Regarding purification strategies, UC emerged as the most frequently employed technique, reported in 5 studies.[52,56–58,63] Several combined approaches were also described, such as UC with microfiltration (MF),[53,54,59] differential centrifugation (DC) with MF,[62] and a multi-step protocol integrating ultrafiltration (UF), MF, and UC.[48] In addition, single-method strategies, including DC,[50,61] MF,[51] UF,[55] and size exclusion chromatography (SEC),[44] were also noted, whereas 2 studies[49,60] did not specify their purification techniques.

Despite their widespread use, current isolation and purification methods often struggle to achieve both high specificity and high recovery, creating challenges for standardization, reproducibility, and scalability in therapeutic applications. This limitation is particularly relevant as interest grows in EV-based drug delivery systems, especially for oncology treatment. To address this gap, there's an urgent need to develop novel approaches that can efficiently produce clinical-grade EVs while maintaining purity and scalability.

All studies included in this review reported EVs with a size distribution typically ranging from 20 to 300 nm, as assessed by various techniques including nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), and atomic force microscopy (AFM). Morphology and membrane integrity were typically assessed by transmission electron microscopy (TEM), scanning electron microscopy (SEM), and AFM. In addition, Western blotting (WB) and flow cytometry (FC) were widely used to detect EV-specific surface and cytosolic markers, while protein concentration was generally measured by the bicinchoninic acid (BCA) assay. The most frequently assessed EV markers included classical identification markers (eg, CD63, CD9, CD81, Alix, TSG101, HSP70, CD3, CD41) and functional markers (eg, CD56, CD44, CD47, CD62, CD82, CD276, CD166, CD274, CD62P, claudins, integrins, EpCAM, E-cadherin). Negative EV markers (eg, calnexin, HSP90, β-actin) were also used in some of the studies to examine the purity of the isolated EV samples and to rule out contamination by other cellular components. As illustrated in Figure 2F, a heatmap was generated based on 17 included studies to visualize the frequency of marker detection. Each value represents the number of studies that reported the corresponding marker. The analysis revealed that classical tetraspanins (CD63, CD81, and CD9) and endosomal markers (TSG101 and Alix) were most consistently identified across studies, highlighting their reliability as canonical EV markers. In contrast, functional surface molecules such as CD44, integrins, and EpCAM showed lower and more variable detection frequencies, suggesting differences in EV origin, isolation approaches, and experimental aims among studies. Appropriate characterization, including quantifying EV content, verifying EV presence, and assessing potential contamination from non-EV components, is essential for the reliability of EV-based research. However, the field continues to face substantial challenges in EVs characterization, primarily due to the lack of universally accepted markers, and EVs naturally vary in size, content, and origin. The Minimal Information for Studies of Extracellular Vesicles (MISEV) 2023 guidelines highlight that no single method can fully characterize EVs, so combining multiple complementary techniques is strongly advised to overcome individual limitations and ensure rigorous, reproducible, and comparable results across studies. Storage conditions for EVs were also examined in this review, revealing that only eight out of 17 studies clearly stated storing EVs at −80 °C before use,[44,50,54,55,59,60,62,63] while the remaining nine did not report any storage details.

It is well recognized that various factors, such as storage temperature, duration, freezing and thawing procedures, storage containers, and the number of freeze-thaw cycles, can significantly affect the structural integrity, composition, and biological activity of EVs. Considering these concerns, the MISEV 2023 guidelines urge researchers to thoroughly

Table 3 Summary of Isolation, Characterization and Purification Methods, Storage Conditions, and Functional Analysis of EVs

First Author	Year	Region	Source	Type of EV	Size Distribution or Mean (±SEM) (nm)	Isolation Method	Purification Method	Characterization Method	Biological Markers	Storage Condition	Adherence to MISEV2023 EVs Characterization Criteria	Adherence to MISEV2023 EVs Purification Criteria
Guo et al[48]	2021	China	E. coli BL21	OMVs	20–250	DC	UF+MF+ UC	NTA, TEM, DLS	—	—	NO	NO
Hashemi et al[49]	2023	Iran	Human NK cells	Exosomes	129.4	Exo-spin™ kit	—	TEM, DLS, WB, FC	CD9, CD63, CD3, CD56, Calnexin	—	YES	YES
Shojaei et al[50]	2021	Iran	ADMSC	Exosomes	40-100	EXOCIB kit	DC	TEM, SEM, DLS, BCA, WB	CD63, CD81	−80°C	YES	NO
Li et al[51]	2020	China	Macrophage	Exosomes	97.3	DC	MF	TEM, DLS, WB	CD63, CD81, Alix	—	YES	NO
Bose et al[52]	2022	USA	4T1 cells	EVs	200-300	DC	UC	NTA, TEM, DLS, Z-Score iBAQ signal	CD9, CD44, CD47, CD81, CD82, CD276, CD166, Claudins, Integrins	—	YES	NO
Y. Ding et al[53]	2023	China	BMSC	sEVs	124.5 ± 86.3	DC	UC+MF	NTA, TEM, WB	—	—	YES	YES
M.J. Haney et al[54]	2020	USA	RAW 264.7	EVs	110.8 ± 4.1	DC	UC+MF	NTA, TEM, DLS, Bradford assay	CD63, TSG101, HSP90	−80°C	YES	NO
K. Zhang et al[55]	2021	China	4T1	ABAs	100-220	Extrusion	UF	TEM, DLS, WB, SDS–PAGE assay	CD47, CD44, EpCAM, E Cadherin	−80°C	NO	YES
D. Uslu et al[56]	2024	Turkey	Human platelets	Exosomes	82.02 ± 5.21	DC	UC	TEM, DLS, WB	CD62, CD9	—	YES	YES
M. Basak et al[57]	2024	India	RAW 264.7	EF	208.7 ± 36.19–257.83 ± 51.06	DC	UC	DLS, WB	CD63, Alix, TSG101, β-actin	—	YES	NO
N. Erwin et al[58]	2024	USA	MDA-MB -231	EVs	—	DC	UC	NTA, TEM	—	—	NO	NO
J. Pullan et al[59]	2022	USA	Raw bovine milk	Exosomes	—	DC	UC+MF	DLS, AFM, FC, TRPS, HRTEM	CD63	−80°C	YES	YES
R. Sarkar et al[60]	2024	India	B16	Exosomes	—	Total Exosome Isolation kit	—	TEM	—	−80°C	NO	NO
Y. Si et al[44]	2022	USA	HEK 293F	EVs	78.2–151.1 (112.3 ± 1.5)	DC	SEC	NTA, WB	CD63, HSP70	−80°C	YES	NO

(*Continued*)

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Table 3 (Continued).

First Author	Year	Region	Source	Type of EV	Size Distribution or Mean (±SEM) (nm)	Isolation Method	Purification Method	Characterization Method	Biological Markers	Storage Condition	Adherence to MISEV2023 EVs Characterization Criteria	Adherence to MISEV2023 EVs Purification Criteria
M. C. Sun et al[61]	2023	China	4T1	EVs	100	Extrusion	DC	TEM, WB	TSG101, CD81	—	YES	NO
Y. W. Wu et al[62]	2023	Taiwan	CPLTs	EVs	120-150	Extrusion, Freeze/thaw cycles, DC	DC+MF	NTA, SEM, DLS, WB	CD41, CD62P, CD9, CD63	−80°C	YES	NO
Z. Zhang et al[63]	2024	China	MSCs	Exosomes	106.1 ± 28.4	DC	UC	NTA, TEM, WB	CD9, TSG101, Calnexin	−80°C	YES	YES

Note: Symbol: "—" indicates data not applicable or not investigated.

Abbreviations: 4T1, murine mammary carcinoma cell line; ABAs, apoptotic body analogues; ADMSC, adipose-derived mesenchymal stem cell; AFM, atomic force microscopy; B16, murine melanoma cell line; BCA, bicinchoninic acid assay; BMSC, bone mesenchymal stem cell; CPLTs, cryopreserved platelets; DC, differential centrifugation; DLS, dynamic light scattering; EF, Exosomal Fragments; EVs, extracellular vesicles; FC, flow cytometry; HEK 293F, human embryonic kidney cell line; HRTEM, high-resolution transmission electron microscopy; HSP70/90, 70/90-kilodalton heat shock proteins; MDA-MB-231, human triple-negative breast cancer cell line; MF, microfiltration; MSCs, mesenchymal stem cells; NK cell, Natural Killer cell; NTA, nanoparticle tracking analysis; OMVs, Outer membrane vesicles; RAW 264.7, murine macrophage cell line; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis assay; SEC, Size-exclusion chromatography; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TRPS, tunable resistive pulse sensing; TSG101, tumor susceptibility gene 101; UC, ultracentrifugation; UF, ultrafiltration; WB, Western blot.

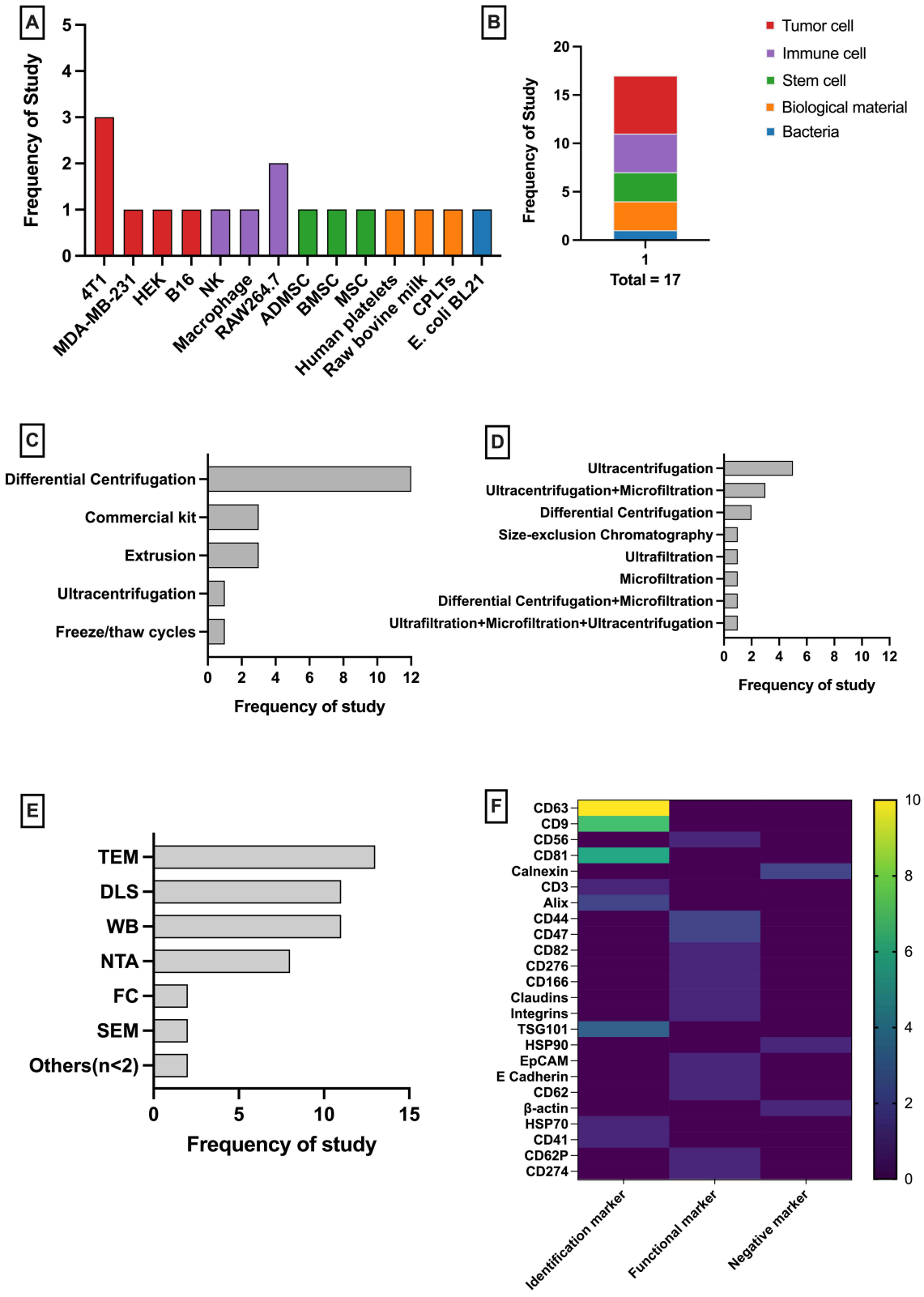

Figure 2 Overview of reviewed studies. (**A**) distribution of source, (**B**) type of source, (**C**) isolation method, (**D**) purification methods, (**E**) characterization methods, and (**F**) common biological markers.

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document all storage-related parameters during EV isolation and preservation, which is crucial for enhancing study reproducibility, transparency, and minimizing storage-induced changes in EV samples.

The reviewed studies demonstrate the wide range of methods used in EV research, with differences in EV sources, isolation techniques, characterization approaches, and storage conditions potentially leading to inconsistent findings and reduced comparability between studies. Therefore, the MISEV 2023 guidelines emphasize the importance of adopting standardized protocols and comprehensive reporting to ensure reproducibility and improve data quality.

Quality Assessment of the Included Study

This section evaluates the overall quality and consistency of the included studies, with a focus on in vivo experimental design, reporting standards, and methodological transparency, as well as adherence to the EV characterization and purity criteria outlined in the MISEV2023 guidelines.

The SYRCLE's RoB tool adopted from Hooijman 2014[72] was utilized to evaluate the risk of bias in the selected studies as shown in Table 4. Among the 11 studies reviewed, 8 (72.7%) explicitly mentioned the randomization of animal groups,[44,48,51,52,54,55,61,63] but 3 (27.3%) did not, resulting in an unclear risk of sequence generation.[53,58,60] The baseline characteristics, such as age, gender, species, and group size, were provided before treatment in 5 studies (27.3%),[48,53,55,60,61] but the remaining 6 studies showed a high risk due to the lack of one or two essential details.[44,51,52,54,58,63] None of the studies disclosed information regarding allocation concealment, random housing, blinding of interventions or caregivers, random outcome assessment, or blinding of outcome assessment; therefore, these potential biases were classified as unclear. Regarding attrition and reporting bias, all studies have a low risk of bias, as they reported complete data and used statistical methods for data analysis. All studies showed low risk of other biases by disclosing ethics approval, potential conflicts of interest, and the roles of collaborating parties. Generally, most of the elements evaluated showed an unknown risk of bias.

In addition to evaluating the in vivo study design, this review also assessed the characterization and purity of EV across all 17 included studies based on the MISEV 2023 guidelines. According to the MISEV2023 guidelines, integrating multiple complementary techniques is recommended for EV characterization, including assessing the quantity and presence of EV and evaluating non-EV components. A detailed summary of adherence to MISEV2023 criteria is provided in Table 3. Specifically, 13 studies fulfilled the essential criteria for EV characterization through the quantitation of EV and identification of at least two positive EV protein markers and non-EV protein markers.

In contrast, four studies lacked sufficient evidence of multiparametric characterization, raising concerns about accurately identifying EVs. Regarding purity, only 6 studies adequately addressed the presence of non-EV components or used recommended orthogonal strategies, while the majority (11 studies) did not meet the MISEV2023 purity requirements. This indicates a subset of studies that consider the potential presence of contaminants. These findings reveal a significant standardization gap in EV research, underscoring the urgent need to raise awareness and improve adherence to standardized guidelines to ensure data reliability and comparability across studies.

Challenges and Future Considerations of EVs as a Drug Delivery System

The landscape of EV-based drug delivery research for TNBC shows a globally expanding yet regionally concentrated trend, with most of the 17 included studies originating from Asia, particularly China, followed by the United States and several European countries, highlighting a strong research focus in Asia and North America, with China and the USA as leading contributors (Figure 3). Temporally, research outputs remained steady from 2020 to 2024, with 3 to 4 studies published annually, suggesting an ongoing development phase rather than a short-term research boom in EV-based therapeutic strategies.

Despite growing preclinical evidence supporting the therapeutic potential of EV-based platforms, major challenges hinder clinical translation, including heterogeneous isolation and purification methods that often fail to achieve both high yield and purity, limiting reproducibility and scalability. Additionally, the lack of universally accepted EV markers and standardized characterization protocols complicates quality assessment, and few studies have examined whether EVs maintain stability and bioactivity during storage and transport. Furthermore, the existing cargo loading methods, such as electroporation and sonication, need to be refined to achieve efficient and controlled cargo loading for maximizing the

Table 4 SYRCLE's Risk Tool of Bias Assessment

Study	Selection Bias			Performance Bias		Detection Bias		Attrition Bias	Reporting Bias	Other
	Random Group Allocation	Baseline Characteristics	Allocation Concealment	Random Housing	Blinding of Intervention and Caregivers	Random Outcome Assessment	Blinding of Outcome Assessment	Incomplete Outcome Data	Selective Outcome Reporting	Other Sources of Bias
Guo et al, 2021[48]	Low risk	Low risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Low risk	Unclear risk	Unclear risk
Li et al, 2020[51]	Low risk	High risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Low risk	Unclear risk	Unclear risk
Bose et al, 2022[52]	Low risk	High risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Low risk	Unclear risk	Unclear risk
Y. Ding et al, 2023[53]	Unclear risk	Low risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Low risk	Unclear risk	Unclear risk
M.J. Haney et al, 2019[54]	Low risk	High risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Low risk	Unclear risk	Unclear risk
K. Zhang et al, 2021[55]	Low risk	Low risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Low risk	Unclear risk	Unclear risk
N. Erwin et al, 2024[58]	Unclear risk	High risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Low risk	Unclear risk	Unclear risk
R. Sarkar et al, 2024[60]	Unclear risk	Low risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Low risk	Unclear risk	Unclear risk
Y. Si et al, 2022[44]	Low risk	High risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Low risk	Unclear risk	Unclear risk
M. C. Sun et al, 2023[61]	Low risk	Low risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Low risk	Unclear risk	Unclear risk
Z. Zhang et al, 2024[63]	Low risk	High risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Low risk	Unclear risk	Unclear risk

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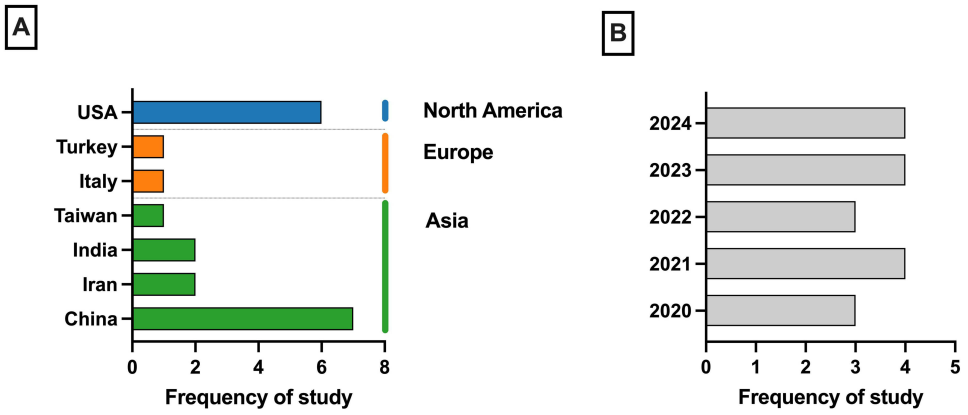

Figure 3 Distribution of review studies for publication by (**A**) region and (**B**) year.

therapeutic potential of EV-based drug delivery systems. Another major challenge is the rapid clearance by the mononuclear phagocyte system (MPS) and the potential off-target accumulation of EV-loaded drugs following systemic administration. Thus, more pharmacokinetics and biodistribution studies in vivo are needed to standardize the in vivo experimental protocol, including the optimal dosing and delivery route of EV-based drug delivery platforms in TNBC. In addition, the inconsistency in reporting of methodologies and outcomes remains a crucial concern. To ensure the transparency and reproducibility of data, it would be essential to adhere to the MISEV 2023 guidelines for standardized reporting. On the translational front, issues such as large-scale production, storage stability, immunogenicity, and biodistribution variability must be systematically addressed to bridge the gap between preclinical research and clinical application. Although some studies explored engineering strategies, such as those modified with targeting ligands or loaded with potent therapeutic agents to enhance delivery precision, the safety and long-term effects of these modifications remain largely unknown.

Future research should focus on harmonizing advanced techniques, developing robust and standardized manufacturing protocols, and expanding multi-center in vivo validations. These steps are crucial for advancing clinical development.

Beyond Drug Delivery – Other Roles of EVs in Cancer Immunotherapy

EVs play a complex role in cancer immunotherapy as they can either promote or suppress tumor progression.[73,74] Their dualistic effects on tumor progression is strongly related to their cellular origin and molecular cargo. Tumor-derived EVs are known to carry immunosuppressive molecules such as programmed cell death 1 ligand 1 (PD-L1),[75–77] FAS ligand (FASLG),[78,79] MHC class I polypeptide-related sequence A (MICA),[80] and TGF-β[81] that can inhibit cytotoxic T lymphocyte activation and differentiation, impair NK cell and dendritic cell function, and promote the expansion of regulatory T cells, thereby fostering an immunosuppressive tumor microenvironment. Through these mechanisms, tumor-derived EVs contribute to immune escape, tumor growth, and resistance to immunotherapy. On the other hand, immune cell-derived EVs (eg, macrophages,[51,54] dendritic cells,[82,83] NK cells[49]) or engineered EVs may deliver anti-cancer agents (eg, miRNAs,[48,50,52,55,57,63] cytotoxic proteins,[55,58] chemotherapy drugs[48,49,54,56,57,59–62]) to activate the immune system and boost the immune response against cancer, thereby sensitizing cancer cells to anti-cancer therapies. Therefore, it is important to understand the complex interplay between the EVs and the tumor for rational design of EV-based therapeutic platforms for the treatment of cancer. A comprehensive consideration of their immunomodulatory effects will be critical to harnessing their full potential in cancer treatment, ie, to maximize anti-tumor efficacy while minimizing the risk of immune-related adverse events.

Conclusion

In conclusion, this review highlights the promise of EVs as a novel drug delivery system for TNBC. Analysis of the included studies demonstrates that EV-based therapeutics enhance the efficacy of anti-cancer agents while minimizing adverse effects. Both in vitro and in vivo evidence support the effectiveness of EV-mediated delivery systems in

selectively targeting TNBC cells. However, challenges such as heterogeneous isolation methods, lack of standardized characterization protocols, and issues with stability and bioactivity during storage hinder clinical translation. Future research should focus on addressing these challenges, adhering to MISEV 2023 guidelines for reporting, and developing robust manufacturing protocols. Ultimately, EV-based drug delivery systems hold significant potential to improve outcomes for patients with TNBC and advance personalized cancer therapy.

Ethics Approval

Ethics approval is not required for this systematic review.

Consent for Publication

All authors read and approved the published version of the manuscript.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest.

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