Journal of Extracellular Vesicles

REVIEW ARTICLE OPEN ACCESS

Methods and Challenges in Purifying Drug-Loaded Extracellular Vesicles

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Received: 31 January 2025 | **Accepted:** 2 May 2025

ABSTRACT

Extracellular vesicles (EV) have emerged as promising nanocarriers for drug delivery. However, the efficient loading of therapeutic molecules into EV and the subsequent purification of drug-loaded EV from unloaded drugs remain significant challenges. This review explores the most used methods for EV purification, meaning the separation of drug-loaded EV from unloaded drugs, including ultracentrifugation, density gradient centrifugation, ultrafiltration, size exclusion chromatography, dialysis and commercial exosome isolation kits. The principles, advantages and limitations of each method are discussed. Critical parameters such as molecular weight cutoff, membrane composition, and the nature of the loaded molecule are highlighted for their impact on the purification process. The review also addresses the technical aspects, including time, cost and equipment requirements, and emphasizes the need for standardized guidelines to improve reproducibility and comparability across studies. By providing a comprehensive overview of current purification strategies, this review aims to guide researchers in selecting the most appropriate methods for advancing EV-based drug delivery systems.

1 | Introduction

Extracellular vesicles (EV) are phospholipid-bilayer vesicles secreted by all cell types, with sizes ranging from 30 to 200 nm. EV contain inherent cargo, including proteins, lipids, nucleic acids and metabolites. They play a key role in intercellular communication (Gurung et al. 2021). Scientific interest in EV as nanocarriers has increased due to their low immunogenicity and biocompatibility, potential for cell-specific targeting, intrinsic cargo and ability to cross biological barriers, distinguishing them from other nanoparticles (Chen et al. 2021; Fu et al. 2020). As previously reviewed, the use of EV as nanocarriers holds great promise in enhancing the efficacy of delivering free molecules (Joshi et al. 2021).

Various molecules, including small chemotherapeutic drugs, proteins, peptides and RNAs, have been loaded into EV, either anchored in the EV membrane or encapsulated within the EV lumen (Du et al. 2023). However, a significant challenge in using EV as nanocarriers is loading relevant amounts of exogenous molecules into them. The intrinsic content of the EV lumen limits the available space for additional exogenous molecules (Meng et al. 2020; Kimiz-Gebologlu and Oncel 2022), and the EV membrane presents a barrier that hinders drug loading. To address these challenges, two strategies are currently employed for incorporating cargo into EV, namely, endogenous and exogenous loading (Sutaria et al. 2017).

Endogenous loading consists of loading the cargo into EV during their biogenesis, followed by isolating drug-loaded EV from the parent cell culture media. Exogenous loading, on the other hand, requires isolating EV from parent cell culture media—or other sources—and subsequently loading the drug via passive

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or active methods. Active loading methods, such as sonication, electroporation or permeabilization, aim to improve drug loading efficiency by temporarily disrupting the EV membrane (Meng et al. 2020). However, loading efficiency is never 100%, and there is often a strong imbalance between the amount of drug successfully loaded into EV and the amount of unloaded drug remaining in the supernatant. Consequently, the removal of unloaded drugs is a crucial step for accurately determining encapsulation efficiency (EE), but also for evaluating the therapeutic potential of drugs associated with EV compared to the free drug (Kimiz-Gebologlu and Oncel 2022).

Hence, while focus is often placed on optimizing isolation and loading methods, the removal of the unloaded drug is a pivotal step in advancing EV as nanomedicines. Finding a suitable purification method for EV that is fast, non-destructive, efficient and reliable is challenging. This review aims to emphasize the importance of the purification process when using EV as drug delivery systems. We present the purification methods that are most commonly used in the literature and explore their advantages and limitations. Moreover, we provide guidance on selecting the most appropriate purification method(s) when loading therapeutic molecules in EV. Studying and optimizing the purification process in the context of EV loading is important for advancing both the understanding and application of EV-based nanocarriers.

2 | The Critical Importance of Purifying Drug-Loaded EV for Drug Delivery Applications

To date, reviews have addressed the challenges of EV-based nanomedicines for clinical applications and translational medicine, such as low loading efficiency, low yield, limitations of large-scale EV production, insufficient targeted delivery, or poor reproducibility (Brezgin et al. 2024; Koh et al. 2023; Kim et al. 2024; Han et al. 2023; Sadeghi et al. 2023). However, the removal of free (unloaded) drugs is not, or only very slightly, discussed in these reviews.

We believe this is an issue, as the adequate evaluation of the effect of a nanomedicine (intended here as the nanoparticle and its loaded content) requires the ability to remove the unloaded drug from the preparation. Failure to remove the remaining unloaded drug from the nanomedicine preparation can lead to biased interpretation of the data obtained during the evaluation of the biological properties of the nanomedicine. Unfortunately, in the context of EV-based nanomedicines, this point is overlooked.

Stability and solubility issues can hinder the use of certain drugs with promising therapeutic effects. Drug-loaded EV often improve bioavailability compared to free drugs by protecting them from degradation and improving solubility (Yuan et al. 2024). If the unloaded drug is present in drug-loaded EV suspensions, it may be degraded or cleared from the body more rapidly, reducing the overall therapeutic effect. Moreover, clinical trials require well-characterized and reproducible formulations with a clear understanding of pharmacokinetics and biodistribution, which differ between free drugs and loaded drugs.

Dose-dependent toxicities and off-target effects limit the clinical applications of some bioactive molecules. Due to the intrinsic homing properties of EV, EV-based nanomedicines enable the administration of lower concentrations of drugs to achieve therapeutic effects. When the free drug is present, it can diffuse non-specifically throughout the body, reducing the efficiency of targeted delivery and potentially causing toxicities. For instance, engineered EV loaded with miRNA have been shown to reduce the general genotoxicity associated with free miRNA (Zhang et al. 2024).

The penetration of some drugs through the blood–brain barrier to achieve therapeutic effects in the central nervous system requires high-dose administration, inducing adverse effects. The ability of EV to cross the blood–brain barrier facilitates drug delivery in the central nervous system. For instance, methotrexate-loaded EV have been shown to enhance crossing through the BBB, substantially increasing the methotrexate concentration within the cerebrospinal fluid while reducing side effects in non-targeted tissues (Zhao et al. 2025).

In addition to the biological effect of nanomedicines, their characterization and quality control are key parameters that should systematically include the assessment of the EE. EE reflects the percentage of drug molecules successfully incorporated into the nanoparticles and is typically assessed by measuring the drug concentration in the drug-loaded EV suspension or by quantifying the amount of unloaded drug remaining in the supernatant after purification.

The separation of unloaded drugs from drug-loaded EV is essential for accurately calculating EE. Quantifying the drug in a mixture containing both free drugs and drug-loaded EV can lead to overestimated EE values, potentially resulting in misleading conclusions regarding the loading capacity of EV and their efficacy as drug delivery systems. We hypothesize that some of the therapeutic effects reported in the literature may have been overestimated due to incomplete removal of the unloaded drug from EV preparations. Taken together, these elements will favour process reproducibility, consistency in the administered dose and adequate data interpretation in the context of EV-based nanomedicines.

3 | Methods Available for the Separation of Drug-Loaded EV From Unloaded Drugs

Currently, the removal of the unloaded drug from EV suspension is performed by using methods similar to those used for EV isolation, such as ultracentrifugation, density gradient centrifugation, ultrafiltration, and size exclusion chromatography (SEC) (Kimiz-Gebologlu and Oncel 2022) (Figure 1). However, there is a lack of standardization and guidelines for selecting the most appropriate method to remove the remaining unloaded drug. The choice should be based on a fair assessment of the pros and cons of each method for a given molecule or family of molecules. In this section, we explain the principle of each method and how it can be used for drug-loaded EV purification. We also discuss the respective advantages and drawbacks of each method.

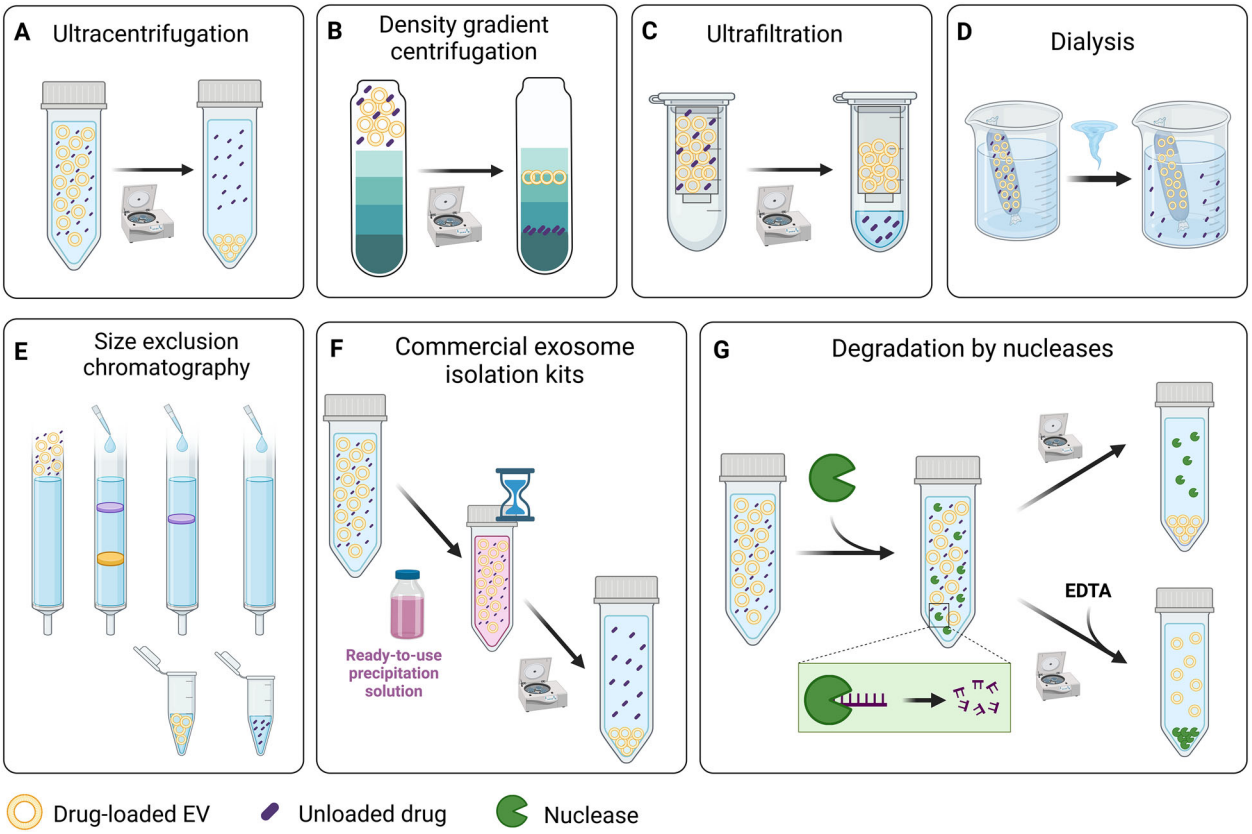

FIGURE 1 | Schematic representation of the different strategies that can be used for the separation between drug-loaded EV and the unloaded drug. Created with Biorender.

3.1 | Ultracentrifugation

Ultracentrifugation is a separation technique that uses centrifugal force to separate particles based on their density, size and medium viscosity. Ultracentrifugation involves significantly higher forces compared to standard centrifugation (>100,000 × *g*), which is necessary to precipitate nanoparticles such as EV (Lin and Qi 2019).

This technique assumes that the density and size of the unloaded drug differ from those of EV. Typically, when EV are denser than the drug, which is mostly the case, a single round of ultracentrifugation at high speed is enough to separate EV and the free molecule. This process concentrates EV in the pellet while the unloaded drug remains in the supernatant. The resulting EV pellet is usually resuspended in a phosphate-buffered solution. Inversely, when the drug is denser than EV, a first step of centrifugation is used to concentrate the unloaded drug in the bottom of the tube while EV are recovered during a second step by using ultracentrifugation (see examples in Table 1, section "low speed centrifugation combined with ultracentrifugation").

For EV collection, rotational speeds and centrifugation times vary between studies, ranging from 100,000 to 170,000 × *g* and 60 to 120 min, respectively.

Ultracentrifugation has been used for separating EV from various types of unloaded drugs, including siRNA, miRNA, proteins or small therapeutic molecules such as doxorubicin or paclitaxel (Table 1).

Based on our research, ultracentrifugation is the most commonly used method so far for EV purification from unloaded drugs. This technique is suitable for large volumes and offers consistent reproducibility (Konoshenko et al. 2018). It enables the resuspension of the EV pellet in a solvent distinct from the one used during the loading process. However, it requires expensive specialized equipment. Besides, the technique has two major drawbacks. The high speeds involved can potentially generate aggregates and damage the EV membrane, leading to drug leakage (Lin and Qi 2019; Konoshenko et al. 2018; Villata et al. 2020; Janouskova et al. 2022). Additionally, particles with densities similar to EV (i.e., protein aggregates, micelles, or drug aggregates) might settle along with EV in the pellet, causing contamination of the formulation with drugs non-associated with EV (Lang et al. 2022; Hendrix et al. 2023).

3.2 | Density Gradient Centrifugation

Density gradient centrifugation is another method for isolating drug-loaded EV. The EV suspension is placed in a tube containing a graded-density centrifugation medium, such as sucrose or iodixanol. Through the application of centrifugal force, drug-loaded EV migrate through the medium, separating from the unloaded drug based on their different densities (Janouskova

TABLE 1 | Strategies used for the purification of drug-loaded EV from unloaded drugs.

Purification method	Source of EV	Loaded molecule	Experimental conditions	Article
Size-exclusion chromatography/gel-filtration chromatography	HEK-293T cells	siRNA	NR	(Faruqu et al. 2018)
	HFL-1 cells	Erastin	NR	(Yu et al. 2019)
	hESCs	cRGDyK	NR	(Zhu et al. 2019)
	M2-type primary peritoneal macrophages	Berberine	Exosome spin columns	(Gao et al. 2021)
	U-87 cells	Paclitaxel	Exo-spin column	(Salarpour et al. 2019)
	Raw milk	Doxorubicin	Sephadex G-25	(Chen et al. 2021)
	Dendritic cells	Paclitaxel	Sephadex G-25	(Wan et al. 2018)
	Raw 264.7 cells	Doxorubicin and paclitaxel	Sephadex G-25 (NAP-10)	(Kim et al. 2016)
	Raw 264.7 cells	Doxorubicin and paclitaxel	Sephadex G-25 (NAP-10)	(Haney et al. 2020)
	HS-5 cells	Pentoxifylline, pyranine	Sephadex G-25 (PD MidiTrap columns)	(Hettich et al. 2022)
	Lemon juice	Doxorubicin	Sephadex G-25 (PD MidiTrap columns)	(Stec et al. 2025)
	Goat milk	Curcumin	Sephadex G-25 (PD-10)	(Albaladejo-Garcia et al. 2024)
	Cow skimmed milk	Curcumin	Sephadex G-25 (PD-10)	(Carobolante et al. 2020)
	BM-MSCs, HUVECs, hESCs, MDA-MB231 cells	Porphyrins of different hydrophobicities	Sephadex G-75	(Fuhrmann et al. 2015)
	Fruits and broccoli	miRNAs	Sepharose CL-2B	(Lopez de Las Hazas et al. 2023)
	PANC-1 cells	PAK4 specific oligonucleotide (siPAK4)	Sepharose CL-2B	(Xu et al. 2021)
	IC-21 cells	Tripeptidyl peptidase-1	Sepharose CL-4B	(Haney et al. 2019)
	Raw 264.7 cells	Catalase	Sepharose CL-6B	(Haney et al. 2015)
	HEK-293T cells	Doxorubicin	Spin desalting columns	(Schindler et al. 2019)
Density gradient centrifugation	EL-4 cells	Curcumin	Sucrose gradient (step gradient: 8%, 30%, 45%, and 60%) 36,000 rpm (rotor NR) for 90 min	(Sun et al. 2010)
	GL-26 cells	Curcumin and activator of transcription 3 inhibitor (JSI124)	Sucrose gradient (step gradient: 8%, 30%, 45%, and 60%) 36,000 rpm (rotor NR) for 90 min	(Zhuang et al. 2011)

(Continues)

TABLE 1 | (Continued)

Purification method	Source of EV	Loaded molecule	Experimental conditions	Article
	BM-MSCs	Curcumin	Sucrose gradient (step gradient: 8%, 30%, 45%, or 60%) 164,000 × *g* for 90 min	(Tian et al. 2018)
	Human red blood cells	Anti-miR-125b and Cas9 mRNA	Sucrose linear gradient (linear gradient: 60%–10%) 150,000 × *g* for 16 h	(Usman et al. 2018)
Low-speed centrifugation	4T1 cells	Sinoporphyrin sodium	6500 × *g* for 25 min	(Liu et al. 2019)
	Bovine milk	Paclitaxel	10,000 × *g* for 10 min	(Agrawal et al. 2017)
	Raw 264.7 cells	Linezolid	10,000 × *g* for 10 min	(Yang et al. 2018)
	Breast cancer cells	S100A4 siRNA	20,000 × *g* for 30 min	(Zhao et al. 2020)
	Red blood cells	RNA	21,000 × *g* for 30 min	(Peng et al. 2022)
Ultracentrifugation	Human plasma	Methotrexate	10,000 rpm for 15 min, three times	(Gul et al. 2024)
	Human PNM and HL60 cells	Piceatannol	100,000 × *g* (time NR)	(Gao et al. 2017)
	U-87 cells	Hydrophobically modified small interfering RNAs (hsiRNAs)	100,000 × *g* for 60 min	(Didiot et al. 2016)
	U-87 MG, bEND.3, PFSK-1, and A-172 cells	Doxorubicin and paclitaxel	100,000 × *g* for 60 min	(Yang et al. 2015)
	HEK-293T cells	miRNA-21	100,000 × *g* for 60 min	(Kim et al. 2020)
	MSCs	Paclitaxel	100,000 × *g* for 60 min	(Kalimuthu et al. 2018)
	Bovine milk	siRNA	100,000 × *g* for 70 min	(Matsuda et al. 2020)
	HEK-293T cells	miR-21i and 5-FU	100,000 × *g* for 70 min	(Liang et al. 2020)
	THP-1 cells	Doxorubicin and cholesterol-modified miRNA 159	100,000 × *g* for 70 min	(Gong et al. 2019)
	M-MSCs	Rapamycin	100,000 × *g* for 70 min	(Song et al. 2025)
	HEK-293T cells	Aptamer F5R2	100,000 × *g* for 90 min	(Ren et al. 2019)
	BM-MSCs	miR-124	100,000 × *g* for 90 min	(Yang et al. 2017)
	Plasma	miRNA (miR-31-5p, and miR-451a)	100,000 × *g* for 120 min	(Pomatto et al. 2019)
	PANC-1 cells	Gemcitabine	110,000 × *g* 70 min	(Li et al. 2020)

(Continues)

TABLE 1 | (Continued)

Purification method	Source of EV	Loaded molecule	Experimental conditions	Article
	Dendritic cells	siRNA	120,000 × *g* for 60 min	(Alvarez-Erviti et al. 2011)
	Dendritic cells	Anti-alpha-synuclein-shRNA-minicircle	120,000 × *g* for 60 min	(Izco et al. 2019)
	Human plasma	Imperialine	120,000 × *g* for 60 min	(Lin et al. 2019)
	MSCs	miR-588	120,000 × *g* for 70 min	(Zhang et al. 2024)
	Dendritic cells	miRNA-140 mimic	120,000 × *g* for 70 min	(Liang et al. 2020)
	Murine neuroblast cell lines or dendritic cells	Cholesterol-conjugated small interfering RNA	120,000 × *g* for 70 min	(O'Loughlin et al. 2017)
	HEK-293T cells	Doxorubicin	120,000 × *g* for 70 min	(Martins-Marques et al. 2016)
	Raw 264.7 cells	SPIONs and curcumin	120,000 × *g* for 90 min	(Jia et al. 2018)
	Primary endothelial cells	siRNA	120,000 × *g* for 90 min	(Banizs et al. 2014)
	MDA-MB-231 and MCF-7 cells	Doxorubicin	120,000 × *g* for 90 min	(Tian et al. 2014)
	HEK-293T cells	Curcumin	120,000 × *g* for 90 min	(Zou et al. 2019)
	Dendritic cells	TGFB1 et IL10	120,000 × *g* for 90 min	(Elashiry et al. 2020)
	MSCs	Doxorubicin	120,000 × *g* for 90 min	(Bagheri et al. 2020)
	3T3 and A549 cells	siRNA	125,000 × *g* for 70 min	(Jhan et al. 2020)
	Monocytes	Bortezomib	130,000 × *g* for 120 min	(Yuan et al. 2024)
	Bovine milk	Curcumin	135,000 × *g* for 90 min	(Aqil et al. 2017)
	Bovine milk	bcl-2 siRNAs	135,000 × *g* for 90 min	(Tao et al. 2020)
	MSCs	miR-210	140,000 × *g* for 90 min	(Zhang et al. 2019)
	LNCaP- and PC-3 cells	Paclitaxel	170,000 × *g* for 120 min	(Saari et al. 2015)
	LL/2 cells	Paclitaxel	170,000 × *g* for 120 min	(Garofalo et al. 2019)
	MDA-MB-231 and HCT-116 cells	Doxorubicin	NR	(Toffoli et al. 2015)
	Blood	Dopamine	NR	(Qu et al. 2018)
	ESCs	Paclitaxel	NR	(Zhu et al. 2019)

(Continues)

TABLE 1 | (Continued)

Purification method	Source of EV	Loaded molecule	Experimental conditions	Article
	Raw milk	miR-31-5p mimic	NR	(Yan et al. 2022)
Precipitation	NR	Curcumin	ExoQuick	(Li et al. 2020)
	Bovine milk	siRNA	ExoQuick	(Aqil et al. 2019)
	MDA-MB-231 cells	Olaparib	ExoQuick	(Jung et al. 2018)
Degradation by nucleases	HEK-293T cells	siRNA	RNase H (10 units, 30 min at 37°C) followed by ultracentrifugation	(Limoni et al. 2019)
	hUCBMNCs, human urine and FBS	miRNA-124-Cy5 and miR-155-5p-Cy3	RNAse (2 μg/mL, 30 min at RT) followed by ExoQuick kit	(de Abreu et al. 2021)
	MSCs	LNA-antimiR-142-3p	RNase H (1 unit) followed by ExoQuick kit	(Naseri et al. 2020)
	Raw 264.7 cells and murine B cells	miRNA-155	RNase H (1 unit) followed by ExoQuick kit	(Momen-Heravi et al. 2014)
	Blood plasma	miRNA-21	RNase followed by ultracentrifugation	(Sun et al. 2020)
	HEK-293T and SKOV3 cells	CRISPR/Cas-9-NLS PARP-1 sgRNA	DNase I, followed by addition of EDTA (20 mM) and centrifugation	(Kim et al. 2017)
	MSCs	siCltc	RNase A	(Chen et al. 2024)
	HEK-293T cells	dsDNA	DNase I (1.5 Kunitz units/μL), followed by addition of EDTA (20 mM) and centrifugation	(Lamichhane et al. 2015)
	HEK-293T and MCF-7 cells	ssDNA	DNase I (1.5 Kunitz units/μL), followed by addition of EDTA (20 mM) and centrifugation	(Lamichhane et al. 2016)
Ultrafiltration	U937 cells	Doxorubicin	10k Da (2000 × *g* for 10 min)	(Goh et al. 2017)
	SKOV3 cells	Triptolide	10 kDa (12,000 × *g* for 30 min)	(Liu et al. 2019)
	Platelet	Epigallocatechin-3-gallate	10 kDa (16,000 × *g* for 30 min)	(Pan et al. 2025)
	EL4 cells	Doxorubicin	30 kDa (3000 × *g* for 15 min)	(Ingato et al. 2018)
	Microglia	Berberine and palmatine	50 kDa (speed and time NR)	(Zhao et al. 2023)
	U-87 cells	Temozolomide	100 kDa (4000 × *g* for 30 min)	(Liu and Zhang 2024)

(Continues)

TABLE 1 | (Continued)

Purification method	Source of EV	Loaded molecule	Experimental conditions	Article
	MSCs	LJM-3064_aptamer	100 kDa (9000 × *g* for 10 min)	(Hosseini Shamili et al. 2019)
	Human adipose-derived mesenchymal stem cells	Methotrexate	100 kDa (2500 r/min for five times)	(Zhao et al. 2025)
	human 293 cells, MCSs, mouse and rat serum	Aptamers	100 kDa (speed and time NR)	(Han et al. 2024)
	HeLa and HT1080 cells	Doxil	100 kDa (speed and time NR)	(Qiao et al. 2020)
	Plasma	Peptides (ILP-CP05, ISP-CP05 and RVG-CP05)	100 kDa (speed and time NR)	(Ran et al. 2023)
	Bovine milk	5-Fluorouracil, Colchicine, Octreotide, Liraglutide	100 kDa (speed and time NR)	(Li et al. 2023)
	Bovine milk	Doxorubicin	100 kDa (speed and time NR)	(Jang et al. 2023)
	HeLa and HT1080 cells	siRNA	100 kDa (speed and time NR)	(Shtam et al. 2013)
	MSCs	Doxorubicin	100 kDa (speed and time NR)	(Gomari et al. 2018)
	Dendritic cells	siRNA/miRNA	100 kDa (speed and time NR)	(Wang et al. 2017)
	Human placentas-derived MSCs	Baricitinib	100 kDa (speed and time NR)	(Tang et al. 2025)
	HEK-293T and MCF-7 cells	RNA	300 kDa (5000 × *g* for 5 min)	(Lamichhane et al. 2016)
	HEI-OC1 cells	Aspirin, arachidonic, eicosapentaenoic, docosahexaenoic, lipoxin A4 and resolvinD1	100 kDa (speed and time NR)	(Kalinec et al. 2019)
	ADSCs	FPG3 or PG3	300 kDa (speed and time NR)	(Ma et al. 2023)
	HEK-293T cells	miR-93-5p	300 kDa (speed and time NR)	(Jeyaram et al. 2020)
	MSCs	Valproïc acid	Cutoff filter NR (5000 × *g* for 10 min)	(Mu et al. 2024)
	Human peripheral blood	miR-21 mimic and miR-21 inhibitor	NR	(Kang et al. 2019)
Dialysis	Blood	Chimeric peptide	NR	(Cheng et al. 2019)

(Continues)

TABLE 1 | (Continued)

Purification method	Source of EV	Loaded molecule	Experimental conditions	Article
External magnetic field	Macrophages (EV-SA-SPIONS)	Doxorubicin	EV conjugated with streptavidin-modified iron oxide nanoparticles collected by magnetic field (free doxorubicin remain in the supernatant)	(Zhang et al. 2017)
Ultrafiltration combined with ultra-centrifugation	Dendritic cells	TGFB1 et IL-10	100 kDa filter tube and ultracentrifugation at 120,000 × *g* for 90 min	(Elashiry et al. 2020)
	TPH-1 cells	Gemcitabine and Deferasirox	100 kDa filter tube and ultracentrifugation at 100,000 × *g* for 60 min	(Zhao et al. 2021)
Low-speed centrifuga-tion combined with ultra-centrifugation	Raw 264.7 cells	Paclitaxel	1000 rpm (rotor NR) for 10 min, and 120,000 × *g*	(Wang et al. 2019)
	HT29 cells and MDA-MB-231 cells	Aspirin	10,000 × *g* for 60 min and 100,000 × *g* for 120 min	(Tran et al. 2019)
	Bovine milk	Withaferin A, bilberry-derived anthocyanidins, curcumin, paclitaxel, and docetaxel	10,000 × *g* for 10 min, and 135,000 × *g* for 120 min	(Munagala et al. 2016)
	Bovine milk	Celastrol	10,000 × *g* for 10 min, and 135,000 × *g* for 120 min	(Aqil et al. 2016)
	Bovine milk	Berry anthocyanidins	10,000 × *g* for 10 min and 135,000 × *g* for 90 min	(Munagala et al. 2017)
Low speed centrifuga-tion combined with ultrafiltration	Bovine milk	Carbamazepine, celecoxib, glimepiride, paclitaxel	Centrifugation at 10.000 × *g* for 10 min then ultrafiltration with 100 kDa filter tube	(Li et al. 2023)

Notes: The purification method, the source of EV as well as the molecule loaded into EV are mentioned. The experimental conditions specific to each study, if known, are indicated.

Abbreviation: NR, not reported.

et al. 2022). EV density varies according to EV content, but is generally considered between 1.1 and 1.19 g/mL (Konoshenko et al. 2018; Brennan et al. 2020).

Only a limited number of articles have reported the use of density gradient centrifugation after drug encapsulation in EV. Often, sucrose is used to form the gradient, with concentrations ranging

from 8% to 60% combined with different centrifugal force and centrifugation times. Both stepwise and linear gradients were used for EV purification (Table 1).

Like ultracentrifugation, this method is costly, laborious, and time-consuming. Moreover, this method is characterized by low yield, and further washing is necessary to remove the density gradient medium from EV suspension (Gandham et al. 2020). As with ultracentrifugation, particles with densities similar to EV can be recovered in the same fraction as EV, resulting in contamination of the EV sample. However, this method stands out for preserving vesicular integrity as it does not require strong centrifugal forces (Wang et al. 2021).

3.3 | Ultrafiltration

This technique uses centrifugation to force the passage of suspension through a porous membrane with a determined cutoff. With this approach, water and molecules smaller than the pores go through the membrane while EV are retained in the concentrate (Sidhom et al. 2020).

Two parameters are critical for an efficient separation by using ultrafiltration. Firstly, the molecular weight cutoff (MWCO) must be correctly chosen to allow the passage of the unloaded drug through the membrane while retaining EV. According to our research, membranes used for EV purification have a wide range of MWCO ranging from 10 to 300 kDa (Table 1). Usually, MWCO of 100 or 300 kDa was used for removing larger molecules including free siRNA or free miRNA (Lamichhane et al. 2016; Wang et al. 2017). Secondly, the composition of the membrane impacts the purification process. EV can bind to membrane fibres, and the degree of binding depends on the nature of the fibres. This binding can reduce the amount of EV collected in the final concentrate. Membranes composed of regenerated cellulose are considered the least retentive compared to hydrosart, polyethersulfone, or cellulose triacetate (Vergauwen et al. 2017).

As compared to ultracentrifugation, ultrafiltration is faster and uses lower centrifugal forces (from 2000 to 12,000 × *g*), which can reduce damage to the EV membrane and, therefore, the risk of drug leakage (Kimiz-Gebologlu and Oncel 2022). This method is relatively simple to implement with a classical cell lab centrifuge (Martins et al. 2023). Nevertheless, ultrafiltration also presents drawbacks. One of the most cited is particle clogging. Suspended particles, such as EV, accumulate on the surface of the ultrafiltration membrane as well as within its pores, which prevents the flow of solvent and, consequently, the passage of unloaded molecules through the membrane (Chen et al. 2018). As far as we know, sterile ultrafiltration units are not commercially available, which increases the risk of contaminating the EV suspension during purification. This presents a challenge when evaluating the biological properties of drug-loaded EV. Finally, even though the volume of the concentrate is reduced after centrifugation, the concentration of the unloaded drug remains the same in both the concentrate and the filtrate. Therefore, multiple washings are required to dilute the free drug as much as possible.

3.4 | SEC

SEC is a column filled with a beads-formed gel that separates particles according to their size. During the elution process, small molecules including the unloaded drug are slowed by entering the pores of the gel, while larger ones, like EV, cannot enter and are eluted earlier from the column. This technique enables the separation of EV, in the first fractions, from the unloaded drug, in the subsequent fractions (Sidhom et al. 2020).

The selection of an appropriate molecular weight cutoff (MWCO), which characterizes the pore size of a column, is critical for an efficient separation. It should be smaller than the molecular weight of EV and larger than the one of the unloaded drug.

The two most commonly bead-formed gels are Sephadex and Sepharose, respectively, made of cross-linked dextran and agarose. Gel filtration chromatography is used for the elimination of various cargos, including oligonucleotides, enzymes, peptides or small therapeutic molecules such as doxorubicin or paclitaxel (Table 1).

Unlike ultracentrifugation or ultrafiltration, SEC uses the passive gravity flow for this application, thus minimizing shear stress effects. This preserves the structure and the integrity of EV, thus reducing the risk of drug leakage (Batrakova and Kim 2015). This technique offers good reproducibility, which is a crucial parameter for drug delivery applications, as it ensures a consistent amount of drug associated with EV from batch to batch. This method is fast and no other equipment is required except the column and eluent, making it relatively affordable (Boing et al. 2014).

Regarding the drawbacks of the approach, Ruysschaert et al. have shown that liposomes, whose structure is close to that of EV, are retained in SEC gel (Ruysschaert et al. 2005). They hypothesize that liposomes are flexible and may be deformed, leading to their passage through the beads and thus their retention. Moreover, separation by SEC results in a diluted EV suspension (Lin and Qi 2019) and co-isolation of similarly sized aggregates (Kimiz-Gebologlu and Oncel 2022). If the unloaded drugs form aggregates with a similar size as EV, they will be slowed in the column and purified in EV fraction. Finally, the EV recovery after elution through SEC is very low (approximately 20% in our hands with an Izon qEV column). Like for ultrafiltration devices, SEC are specifically designed for EV isolation and are not sterile, and cannot be autoclaved.

3.5 | Dialysis

This method consists of eliminating the free drug through a semi-permeable membrane. Typically, the sample containing drug-loaded EV and the free drug is loaded in a dialysis cassette equipped with a semi-permeable membrane of a specific cutoff immersed in a dialysis medium (usually PBS or another buffer) under mild agitation. The unloaded drug diffuses across the membrane under the force of the concentration gradient, while larger molecules (like EV) are not able to pass through (Lin and Qi 2019).

Although dialysis is a widespread purification method for nanoparticles such as liposomes, only a few numbers of articles report dialysis as a method to separate unloaded drugs from drug-loaded EV, probably due to the challenges encountered with this method (Cheng et al. 2019). Indeed, dialysis requires rigorous controls and adjustments. Firstly, determining the appropriate dialysis time is crucial for effectively removing the unloaded drug while preserving drug loading. Short dialysis times can prevent the complete elimination of the unloaded drug, while extended times can initiate a release of the loaded drug (Sun and Du 2012). Indeed, dialysis is a widespread method for studying drug release in vitro (Zhao et al. 2023). Secondly, the MWCO of the membrane must be selected based on the size of the EV and the drug: it should retain EV inside the dialysis bag but allow free diffusion of the unloaded drug. Thirdly, the selection of the dialysis medium plays a crucial role in this process. Parameters such as volume, temperature, and pH of the dialysis medium need to be optimized (Sun and Du 2012). The drug should be soluble in the dialysis medium to avoid the formation of aggregates that would not cross the membrane. PBS is commonly used as a dialysis medium, but may not be suitable for poorly soluble or lipophilic drugs (Lin and Qi 2019).

This simple and cost-effective method can process large volumes and is relatively gentle in removing the unloaded drug due to the concentration gradient used (Sun and Du 2012). However, this method requires long processing times and a regular replacement of the dialysis medium to maintain the concentration gradient between the EV suspension (within the cassette) and the dialysis medium (outside the cassette) (Lin and Qi 2019). Larger molecules, such as proteins, have lower diffusion coefficients, limiting their passage through the semi-permeable membrane. Moreover, optimizations to determine the most appropriate dialysis time and dialysis medium must be carried out for each experiment.

3.6 | Commercial Exosome Isolation Kits

Commercial isolation kits like ExoQuick rely on a polymer that gently precipitates exosomes and microvesicles (De Sousa et al. 2023). These kits use volume-excluding polymers to reduce the solubility of EV and similarly sized proteins and particles, which are subsequently isolated using low-speed centrifugation.

The sample containing EV and the free drug is incubated with the ready-to-use precipitation solution for 30 min, 1 h, or overnight, depending on the kit, at 4°C, followed by a centrifugation step to pellet the EV/polymer suspension. Generally, the companies selling the kits do not disclose details of the principles of EV isolation.

Although not the most commonly used, commercial isolation kits such as ExoQuick have been reported in articles for separating loaded EV from unloaded molecules such as nucleic acids, curcumin and olaparib (Table 1). This technique is readily accessible, less labour-intensive, and does not require specialized equipment. In addition, it allows high EV recovery (Lang et al. 2022). However, commercial isolation kits are relatively expensive, and the analysis is limited to a small number of samples (De Sousa et al. 2023). As manufacturers do not disclose the exact chemical composition and mechanism of the polymers used in these kits, it is challenging for researchers to understand or control the purification process fully. This can result in variability across experiments and difficulty interpreting results, particularly if unintended interactions occur between the polymers and the sample components (Welsh et al. 2024). Another notable constraint of ExoQuick is its lack of specificity, which can lead to the co-isolation of non-exosomal particles/molecules alongside the desired drug-loaded EV (Tang et al. 2017).

3.7 | Degradation by Nucleases

This purification strategy is specific to the loading of nucleic acids into EV. Several studies have reported the use of ribonucleases (RNAse) or deoxyribonucleases (DNAse) to degrade unloaded RNA and DNA, respectively, making it a promising strategy to ensure the complete elimination of unloaded nucleic acids. For example, RNAse treatment is commonly used to remove contaminating external RNA when analysing the RNA content within EV (Mateescu et al. 2017). It is important to note that the EV-associated RNA, that is, RNA adsorbed on the EV outer surface, could be degraded by these nucleases (Rankin-Turner et al. 2021). Therefore, this approach is preferable for the isolation of EV with only RNA/DNA stably incorporated into their core.

Degradation by nucleases is not systematically applied after RNA or DNA encapsulation into EV. Some papers have opted for more conventional purification methods such as SEC (Faruqu et al. 2018), precipitation via a commercial kit (Aqil et al. 2019) ultracentrifugation (Zhang et al. 2019; Banizs et al. 2014), or ultrafiltration (Wang et al. 2017). Lamichhane et al. investigated the loading of siRNA, miRNA, and ssDNA into EV. They used DNAse I to remove ssDNA, but avoided using RNAse for miRNA removal due to difficulties in successfully inactivating RNAse A, opting for ultrafiltration instead (Lamichhane et al. 2016).

EV are typically incubated with the nuclease for 30 min at RT or 37°C (Table 1). However, incubation times and temperatures are not consistently reported across studies. Two distinct protocols are described in the literature. In the first one, EV are incubated with the nuclease, followed by ultracentrifugation or precipitation to collect RNA/DNA-loaded EV in the pellet (Limoni et al. 2019). In the second one, EV are incubated with the nuclease, which is subsequently deactivated by adding EDTA (20 mM), followed by centrifugation to pellet the inactivated enzyme and digested unloaded RNA/DNA (see examples in Table 1, in section "degradation by nucleases").

This technique is easy to use, low-cost and less labour-intensive. It is suitable for large volumes. Moreover, even if some unloaded nucleic acids remain in the EV suspension after centrifugation, they are inactivated by the nucleases, minimizing the risk of observing therapeutic effects caused by unloaded molecules. No information on EV recovery was found in the literature. If drug-loaded EV are recovered using ultracentrifugation, it may result in the same drawbacks described in the ultracentrifugation section, such as the formation of aggregates and potential damage to the EV membrane.

4 | Parameters to Consider When Choosing a Purification Method

When exploring EV as drug delivery systems, the main consideration is typically on selecting suitable EV isolation and loading methods. However, there is a notable lack of literature on purification strategies that separate unloaded drugs from drug-loaded EV after the loading process. This purification process is a fundamental but often neglected aspect, as evidenced by the lack of reviews dedicated to this topic.

Various parameters must be considered when choosing a purification method, including the number of EV recovered after purification, the loading method, the nature of the loaded molecule, the isolation method, the cost-effectiveness and the convenience of use. These factors could significantly influence the success and feasibility of experiments. Further investigations in this direction hold promise for developing standardized protocols or guidelines for EV purification.

4.1 | EV Recovery

When selecting a separation method for drug-loaded EV, a crucial factor to consider is the yield of EV recovery after purification. Notably, a limitation in the advancement of EV-based nanocarriers for clinical applications is the low yield of EV isolated from parent cells or tissues (Chen et al. 2021). Therefore, minimizing EV loss during the purification process is essential for progressing toward in vivo pre-clinical studies and, more significantly, for clinical trials.

EV recovery differs depending on the purification method employed. Two common shortfalls are found in many papers. Firstly, data are absent regarding the quantity of EV both pre- and post-purification. Secondly, EV recovery is mostly estimated by measuring the protein concentration using Bradford assay, while the protein content varies according to the EV source. Therefore, the protein concentration does not correlate with the particle number. Consequently, comparing methods and data across publications to identify the methods with the highest EV recovery is complex. However, comparative studies on EV isolation methods are common and can provide some guidance, as isolation methods are quite similar to purification methods. For instance, the literature suggests that ultracentrifugation and gradient density centrifugation result in a lower yield of EV compared to SEC (Du et al. 2023; Xu et al., 2023).

4.2 | EV Purity

A second factor to consider in the purification process of EV is ensuring their purity, specifically the complete removal of any unloaded drug. Effective separation of drug-loaded EV from unloaded drugs is crucial for ensuring enhanced stability, reduced side effects, targeted delivery and accurate determination of EE.

For example, if the free molecule forms aggregates or micelles similar in size to EV, purification methods based on size exclusion may be insufficient, necessitating alternative methods to prevent co-isolation. When using ultrafiltration, membrane saturation can occur due to the accumulation of EV or unloaded drugs on the membrane surface and within its pores. This can prevent solvent flow and thus the passage of free drug molecules through the membrane (Chen et al. 2018).

Directly related to this issue, the inability of current purification methods to separate unloaded EV from loaded EV poses an additional challenge. When incubating EV with a drug, it is uncertain whether each vesicle will successfully load the drug, even when physical or chemical methods are used to disrupt the EV membranes. Consequently, most studies probably work with a heterogeneous mixture of EV, some of which contain the drug while others do not. In cases where EV have intrinsic therapeutic properties, such as MSC-derived EV, the administration of unloaded EV can be beneficial (Wiklander et al. 2019). However, ensuring the safety of EV used as drug delivery vectors is crucial. For instance, the potential oncogenic risk of tumour cell-derived EV, often employed to enhance cancer cell targeting, must be carefully considered (Koh et al. 2023).

4.3 | Loading Methods

As mentioned in the introduction, two strategies exist for loading drugs into EV: endogenous and exogenous loading. Endogenous loading (Figure 2A) presents the advantage of combining EV isolation and purification into a single step. Conventional isolation methods such as ultracentrifugation, SEC, or ultrafiltration are used to collect EV, and simultaneously eliminate the unloaded cargo. Opting for endogenous loading instead of exogenous loading can reduce EV losses and minimize EV damages since the isolation and purification occur in a single step. However, this method is limited to molecules compatible with the cellular machinery of EV-producing cells and exhibit low cytotoxicity.

In contrast, exogenous loading (Figure 2B) implies isolating EV before the cargo loading and an additional post-loading purification step to remove any unloaded drug. This two-stage approach involves potential losses of EV at each stage, suggesting higher overall losses. However, endogenous loading has shown lower efficiency than exogenous loading, which can enhance loading efficiency by using physical and chemical methods to disrupt EV membranes (Sutaria et al. 2017). The aforementioned limitations regarding the effective removal of unloaded molecules apply to both endogenous and exogenous methods.

4.4 | Nature of the Loaded Molecule

The nature of molecules loaded into EV is an important parameter to consider when selecting a purification method. Molecules encapsulated into EV differ in terms of size, charge, and solubility. The purification method should thus ensure the absence of unwanted interactions with the drug. The mismatch between the purification method and the physicochemical properties of the drug is a significant issue.

Haney et al. explored the ability of macrophage-derived EV loaded with low molecular chemotherapeutic agents—specifically, doxorubicin and paclitaxel—to target cancer cells. Different loading methods have been carried out for each

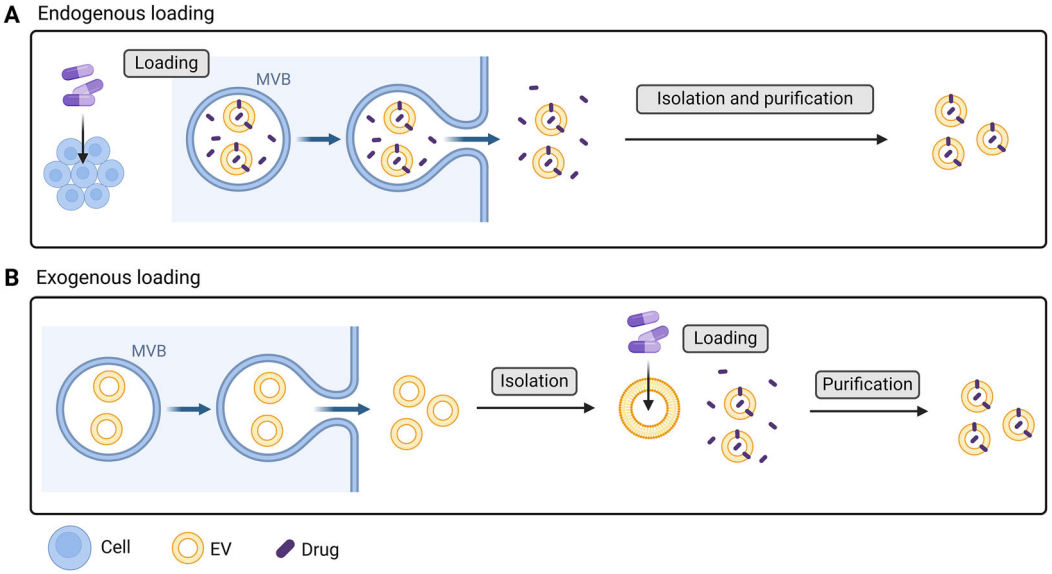

FIGURE 2 | Schematic representation of the two strategies that can be used to load drugs into EV. (A) Endogenous loading consists of loading the drug into EV during their biogenesis, followed by isolation and purification of drug-loaded EV from the parent cell culture media. (B) Exogeneous loading consists of isolating EV from parent cell culture media, then loading the drug into EV, and finally purifying drug-loaded EV. Created with Biorender.

molecule due to their different solubility in water solutions (doxorubicin is a hydrophilic molecule while paclitaxel is a hydrophobic one). Even though distinct loading methods were tested for preparing doxorubicin-loaded EV and paclitaxel-loaded EV, the separation of unloaded drugs from the drug-loaded EV was carried out in both cases by SEC (using a NAP-10 Sephadex G25 column) (Haney et al. 2019). However, the behaviour of doxorubicin and paclitaxel molecules during elution through the SEC is likely to be different. SEC requires hydrophilic eluents, mostly PBS, which is not compatible with insoluble or lipophilic drugs, like paclitaxel. It could lead to the formation of aggregates/precipitates that are similar in size or larger than EV, and consequently could be co-isolated with drug-loaded EV (Lin and Qi 2019).

Another study investigated the loading efficiency of porphyrins with varying degrees of hydrophilicity (Fuhrmann et al. 2015). The purification of porphyrin-loaded EV was conducted by SEC using Sephadex G75, regardless of the porphyrin nature. The purification method was not adjusted to account for the differences in solubility between the porphyrins. The researchers hypothesized that variations in the loading efficiency of porphyrins into EV were influenced by their hydrophobicity. However, the effectiveness of separation between unloaded drugs and drug-loaded EV may also depend on the nature of the porphyrin. Consequently, one could hypothesize that the removal of unloaded drugs is incomplete and varies depending on the nature of the porphyrins, potentially leading to an overestimation of the loaded porphyrin and a bias in interpreting the loading efficiency.

To the best of our knowledge, only one study adapted the purification method based on the hydrophobicity of the cargo. Unloaded hydrophilic drugs were removed by ultrafiltration, while unloaded hydrophobic drugs were separated from EV using low-speed centrifugation combined with ultrafiltration. However, the rationale behind this choice was not explained (Li et al. 2023). One hypothesis could be that hydrophobic molecules form aggregates in aqueous solvents and the use of lower speed during the initial centrifugation step enables the heavier drug aggregates to be selectively separated first. It should be noted that the combination of low-speed centrifugation and ultracentrifugation has been used by several papers for removing lipophilic molecules (Table 1, section "low-speed centrifugation combined with ultracentrifugation"). Only one of them (Tran et al. 2019) explicitly mentions using low-speed centrifugation (10,000 × *g* for 1 h) to remove aggregates and unloaded drugs, followed by ultracentrifugation to pellet drug-loaded EV.

Furthermore, the purification of lipophilic molecules does not consistently involve low-speed centrifugation. Single-step ultracentrifugation has been used in some studies to remove paclitaxel, curcumin, imperialine or piceatannol (Table 1). This technique also requires aqueous solvents, in which hydrophobic molecules may form precipitates that make purification more complex.

Another challenge is the diversity of methods found in the literature for the removal of a specific drug. For instance, the purification method employed to separate free-doxorubicin from doxorubicin-loaded EV varies from one study to another: SEC (Chen et al. 2021), ultracentrifugation (Martins-Marques et al. 2016; Tian et al. 2014; Bagheri et al. 2020), ultrafiltration (Gomari et al. 2018; Ingato et al. 2018), or external magnetic field (Zhang et al. 2017) have been used. Additionally, when ultrafiltration is used, varying MWCO are applied, even for the same molecule. For instance, MWCO ranging from 10 to 100 kDa have been used to purify unloaded doxorubicin (Goh et al. 2017; Jang et al. 2023). This variability in purification methods complicates the comparison of results across studies.

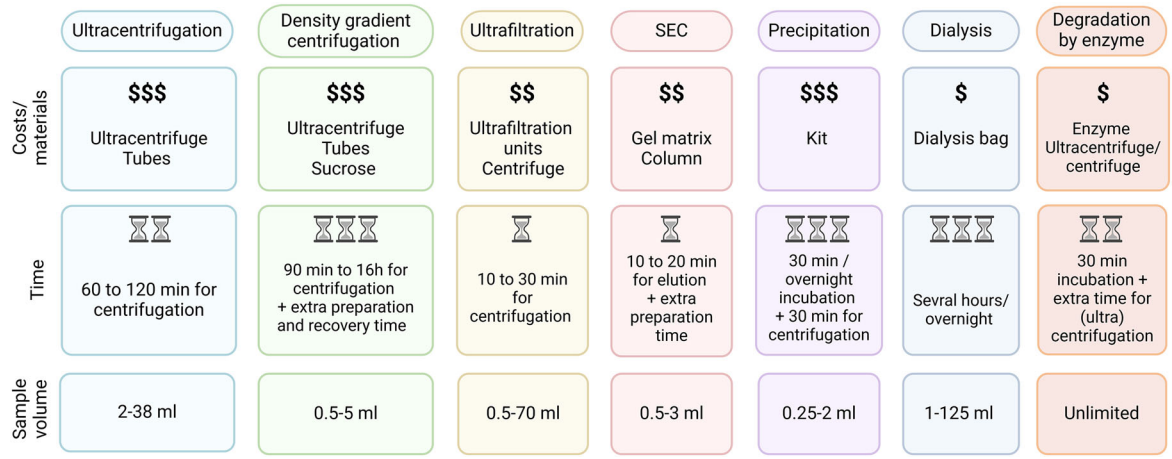

FIGURE 3 | Costs, required materials, handling time, and sample volume for seven different purification methods. At the moment of writing, 1 EUR (€) corresponds to 1.12 USD ($) and 7.9 CNY (¥). Created with Biorender.

4.5 | Technical Aspects

Various technical aspects such as time, costs and equipment, as well as the sample volume, must be considered when choosing a purification method (Figure 3).

Although the passage of the sample through the SEC takes only a few minutes, additional steps to (Gurung et al. 2021) prepare the SEC by hydrating the resin and placing it in an appropriate tube, as well as (Chen et al. 2021) washing the SEC before and after sample passage, add extra time to the process. The density gradient centrifugation takes 90 min (for step gradient) to 16 h (for linear gradient), which corresponds to the centrifugation time. However, additional steps, such as preparing the density gradient and collecting fractions after centrifugation, extend the handling time. Ultracentrifugation takes from 60 to 120 min (Table 1). Precipitation via kits varies between 30 min and an overnight incubation (depending on the kit supplier), followed by 30 min of low-speed centrifugation. Ultrafiltration typically ranges from 10 to 30 min. Dialysis usually takes many hours, even overnight. Finally, incubation of EV with RNAse or DNAse lasts 30 min, followed by additional time for collecting drug-loaded EV and removing the enzyme by centrifugation, ultracentrifugation, or precipitation.

The handling volume varies depending on the purification method. Dialysis and ultrafiltration allow for processing larger sample volumes than SEC or precipitation (Figure 3). The limited handling volume of some methods prevents their use for large-scale production.

Ultracentrifugation and density gradient centrifugation require costly specialized equipment, potentially limiting access for some research teams (Martins et al. 2023; Xu et al. 2023). An ultracentrifuge typically ranges in cost between €20,000 and €100,000. However, once acquired, they are economical methods, costing approximately €7 per tube and €0.1 per gram of sucrose. The cost of ultrafiltration ranges from €5 to €40 per unit. On average, a column of SEC costs around €15. Purchasing bulk gel matrix is more cost-effective than purchasing packed columns such as Izon qEV. For EV isolation kits, the average cost is €30 per reaction, depending on the specific kit (one reaction being for 0.25–2 mL of sample). Dialysis bags are available in membrane tubing form, priced at €0.24/cm of length (sufficient for 2–10 mL of sample per cm), or in cassette form, which is easier and safer to handle but more expensive, at €14 per cassette, accommodating volumes ranging from 1 to 125 mL. RNAse H varies from €2/unit to €7/unit, depending on the supplier. DNAse I costs approximately €0.1/unit.

5 | How Can the Purification Process be Improved?

The purification process of drug-loaded EV is a critical aspect of their use as nanocarriers for drug delivery. Although the literature may not extensively cover this topic, several avenues need to be explored for selecting the appropriate purification method depending on each application. In this section, we discuss some promising ideas, such as comparing purification methods within the same study, combining different purification methods, using an affinity-based method, and adhering to guidelines.

5.1 | Comparison of Different Purification Methods to Determine the Optimal Compromise for Each Specific Application

Considering (Gurung et al. 2021) the absence of a standardized method for EV purification and (Chen et al. 2021) the necessity for the purification method to be tailored to the study/drug, it could be interesting to compare different purification methods within individual experiments to identify the most suitable and effective approach. Unfortunately, comparative studies evaluating various EV purification methods are rare. As far as we know, there is only one such study (Chen et al. 2021). The authors have evaluated the recovery of doxorubicin-loaded EV after purification using SEC or ultrafiltration. Their study highlighted that ultrafiltration, specifically using Vivaspin 20 Diafiltration device (Sartorius) with 300 kDa MWCO, resulted in a significant loss of drug-loaded EV compared to SEC. This could be attributed to the adsorption of drug-loaded EV onto the membrane filter (Liu et al. 2022).

However, these results are specific to this particular experiment, and additional studies are needed to determine whether they can be generalized beyond doxorubicin-loaded EV.

5.2 | Combination of Different Purification Methods

Several studies have reported the use of combined purification methods to remove unloaded drugs through complementary mechanisms (Table 1).

One common approach, mentioned in several articles, is the combination of low-speed centrifugation followed by ultracentrifugation. As discussed earlier, low-speed centrifugation at 10,000 × *g* is likely used to remove debris and drug aggregates, and subsequent ultracentrifugation at speeds of 100,000 × *g* or higher to pellet EV. This approach has been consistently used in studies involving the loading of hydrophobic cargo (Table 1, section "Low-speed centrifugation combined with ultracentrifugation"). However, an important cautionary note is that the approach presupposes that all drugs aggregate, which may not always be true.

Elashiry et al. and Zhao et al. combined ultrafiltration with ultracentrifugation, but no explicit justification is mentioned to support that choice (Zhao et al. 2021; Elashiry et al. 2020). Most likely, ultrafiltration was used to concentrate the sample (and remove molecules smaller than the cutoff) before using ultracentrifugation to collect EV in the pellet.

Lamicchane et al. used ultrafiltration followed by an incubation with DNA I enzyme for 20 min to remove the excess of unloaded DNA (Lamichhane et al. 2015).

The combination of several methods increases the risk of EV loss and drug leakage from EV, but has the advantage of providing EV with high purity, that is, fewer contaminating unloaded drugs. Additionally, combining methods increases experimental complexity and associated costs.

5.3 | Enhance the Selectivity by Using an Affinity-Based Method

The methods discussed above are primarily based on the size and density of EV. In contrast, affinity-based techniques use highly selective and specific interactions between markers present on the EV membrane, such as tetraspanins, heparin, heat shock proteins or phosphatidylserine, and their corresponding ligands (Nakai et al. 2016). The ligands against their surface markers are immobilized on magnetic beads, plates, microfluidic devices or chromatographic stationary phases.

The main advantage of this method is the possibility, in theory, to purify only EV without drug aggregates. This is achieved by selecting a ligand that interacts with a surface molecule on EV, but not with the unloaded drug. However, this method is limited by the lack of reliable markers and by the heterogeneous expression of EV markers, even among EV originating from the same source (Gutierrez-Millan et al. 2021). Additionally, detaching bound EV from ligands using mild elution conditions poses a significant challenge. The use of alkaline or acidic elution buffers, which are often required to efficiently release EV during the elution process, may compromise their integrity and functional activity. This presents a potential drawback to the method, as maintaining the biological function of EV is crucial for their therapeutic application (Strohle et al. 2022).

The affinity-based method is not used for drug-loaded EV purification, likely due to the drawbacks mentioned above and the need for an in-depth understanding of the interactions between EV molecules and ligands.

5.4 | Adherence to Guidelines

Purification guidelines need to be proposed to enhance understanding and comparability across studies. Rankin-Turner et al. have published guidelines specifically addressing EV loading (Rankin-Turner et al. 2021). They recommended adding a detailed method used for the purification of drug-loaded EV, including centrifuge model and rotor type, speed and time of centrifugation, the number of wash/filtration steps, and the type of filter used. Furthermore, they recommended quantifying EV after the purification of drug-loaded EV to establish EV loss during the purification process. They also consider it important to specify whether the loading efficiency was calculated by an indirect method (measurement of the remaining unloaded drug the supernatant/eluate after isolation of drug-loaded EV) or by a direct method (measurement of the drug in EV suspension after destruction of drug-loaded EV to release the drug) (Rankin-Turner et al. 2021). Systematically including this detailed information could improve data transparency, thus improving reproducibility and facilitating relevant comparisons across studies. Sharing of knowledge and findings, whether positive or negative, could help advance research in this field.

6 | Conclusion

The investigation and the selection of a suitable purification method are critical but often overlooked in the field of EV-based drug delivery systems. The lack of dedicated reviews and studies focusing on purification strategies contributes to the complexity of selecting the method to isolate drug-loaded EV. Only effective removal of the unloaded drug will enable accurate quantification of the loaded drug into EV and will ensure that the observed therapeutic effect is due to the loaded drug rather than the residual unloaded drug. Since purification methods can affect the quality and the yield of drug-loaded EV, choosing the appropriate approach can significantly impact the experimental outcomes. Establishing and following guidelines would improve transparency, reproducibility, and comparability across studies. Further research and more effort could lead to the development of robust protocols and guidelines, ensuring the successful and consistent purification of drug-loaded EV in a variety of experimental contexts.

Author Contributions

Marie Auquière: Writing – original draft (lead). **Giulio G. Muccioli**: Writing – review and editing (equal). **Anne des Rieux**: Writing – review and editing (equal).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study

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