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REVIEW

Plant-Derived Nanovesicles: Resolving Conceptual Confusion, Overcoming Isolation Challenges, and Advancing Translational Potential

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Abstract: Plant-derived nanovesicles (PDNVs) are promising bioactive nanoparticles with potential in drug delivery, immune regulation, and tissue repair. However, inconsistent terminology and isolation methods hinder reproducibility and clinical translation. A key confusion lies in their comparison with plant extracellular vesicles (PEVs), which are naturally secreted, whereas PDNVs are typically extracted by breaking plant tissues. This process yields a mix of extracellular and intracellular vesicles, creating both functional diversity and challenges. The heterogeneity complicates standardization, large-scale production, and quality control. Here, we clarify the distinctions between PDNVs and PEVs, and then we explore key factors that affect PDNVs isolation. These include the type of plant used, how the plant is processed, and how the vesicles are purified. We highlight workflow-specific optimizations that boost PDNV recovery (e.g. 4–5 fold higher yields with optimized PEG precipitation) and enhance purity (e.g. ATPS delivering multi-fold higher recovery while eliminating >95% of protein contaminants). Finally, we propose strategies to help establish standardized methods for using PDNVs in biomedical applications.

Keywords: plant-derived nanovesicles, plant extracellular vesicles, isolation methods, plant pretreatment, clinical translation

Introduction

In recent years, PDNVs have attracted considerable attention as a promising class of bioactive nanoparticles. Derived from plant sources, these vesicles exhibit strong potential across multiple therapeutic domains.[1] Their natural origin confers several advantages, including high biocompatibility, low immunogenicity, and the capacity to carry a diverse range of plant-derived bioactive compounds.[2] Owing to these properties, PDNVs are under active investigation for applications in drug delivery,[3] immunomodulation,[4] and tissue repair.[5] Studies have explored their therapeutic potential in cancer treatment,[6] inflammatory disease management,[7] and regenerative medicine.[8] Since the first report on plant exosome-like vesicles in 2009, related publications have increased rapidly, with an average annual growth rate of approximately 29.26%, signaling strong momentum in the field and underscoring the need for aligned standards.[9] Despite over a decade of research and increasing publications, the field lacks the standardized nomenclature that enabled clinical translation of mammalian EVs. In parallel, the potential scalability and cost-effectiveness of plant-based vesicle production has attracted significant commercial interest, making standardization increasingly urgent. Yet, clinical progress remains modest, and expectations for regulatory-grade manufacturing and quality control, already well established in the broader EV community, make it important to define fit-for-purpose isolation and characterization strategies at an early stage.[10]

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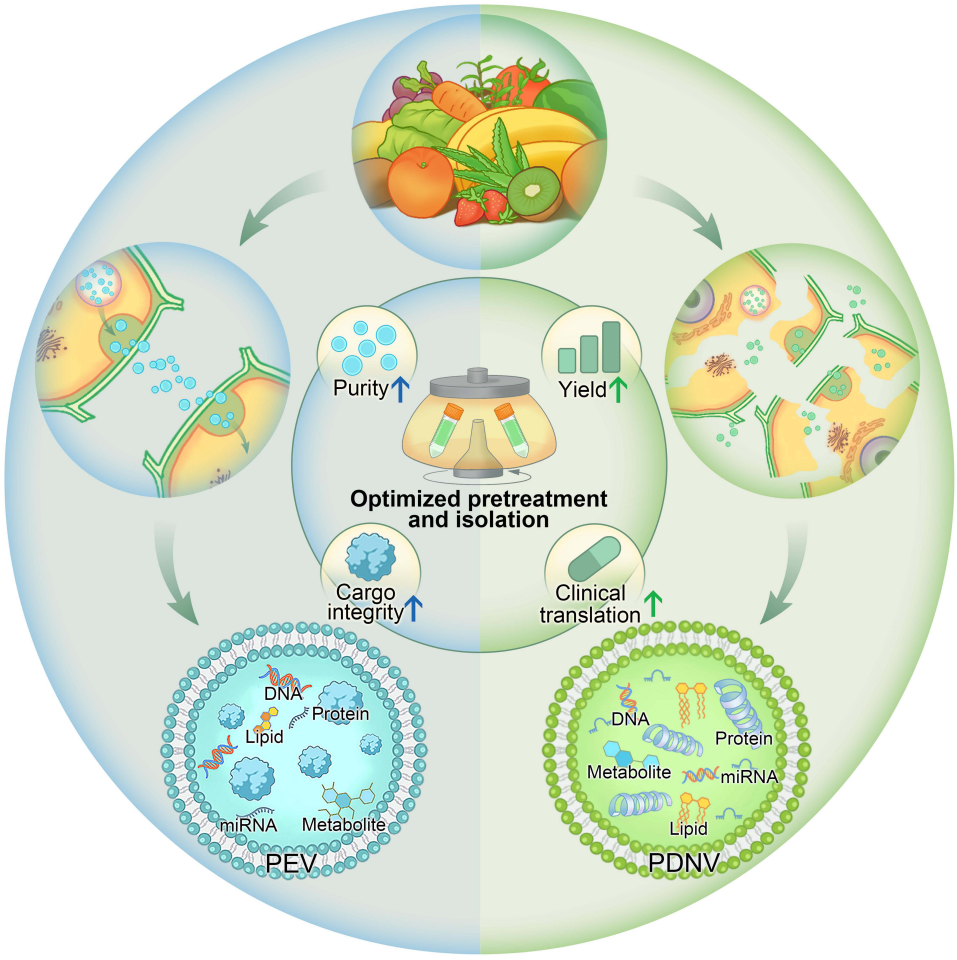
Graphical Abstract



Despite growing interest, the field faces a critical conceptual and methodological challenge: the lack of a clear definition of PDNVs. This ambiguity has led to inconsistencies that hinder research quality and impede clinical translation. A major source of confusion lies in the frequent conflation of PDNVs with PEVs. Although often used interchangeably, these vesicle types differ fundamentally.[11] PEVs are secreted through natural, non-destructive processes and are localized in the plant apoplastic space.[12] In contrast, PDNVs are typically obtained by mechanically disrupting whole plant tissues, resulting in a heterogeneous mixture containing extracellular vesicles (EVs), intracellular components, and various non-vesicular particles.[13] Historically, ultrastructural observations of vesicle-like structures in plant cells date back to the 1960s.[14] However, the field gained momentum only after reports around 2009 describing exosome-like vesicles isolated from plant tissues, followed by a rapid expansion of related applications and protocols.[15] Definitions did not keep pace. As a result, different preparation types were often discussed under shared umbrella terms, which is a big reason the conceptual confusion persists today.

Terminological inconsistency further complicates the field. Researchers have employed overlapping and ambiguously defined labels such as "plant-derived exosome-like nanovesicles[16]", "plant-derived nanoparticles[17]", "plant-derived exosomes[18]", and "extracellular vesicle-like particles".[19] These terms are rarely used with precision or consistency, leading to substantial variability in isolation procedures, compositional profiles, and reported biological activities.[20] This confusion is amplified by the way methods are evaluated and reported. Across protocols, benchmarking is frequently incomplete, and key information is described in a patchwork manner. Purity assessment may be detailed in one study but barely addressed in another. Evidence for particle identity can vary from minimal to fairly rigorous. Contaminants are

sometimes acknowledged only in passing. This lack of standardization hampers inter-study comparisons and slows the translation of PDNV research into clinical practice.[21]

To address these challenges, the field urgently requires a unified nomenclature and standardized protocols for the isolation and characterization of PDNVs. A framework analogous to the MISEV (Minimal Information for Studies of Extracellular Vesicles) guidelines established in mammalian extracellular vesicles research may provide a valuable foundation. In mammalian EV research, community-driven consensus standards including minimum information reporting, fit-for-purpose characterization, and transparent description of isolation trade-offs, have helped mitigate similar issues of heterogeneity and methodological variability, offering a practical precedent for the PDNV field.[22,23]

PEVs and PDNVs—the Same Juice? Not Quite

Definitions and Biogenesis Pathways

PDNVs and PEVs originate through fundamentally distinct biological processes. PEVs are actively secreted by living plant cells into the apoplastic space via exocytosis. This secretion mechanism closely resembles the biogenesis of EVs in mammalian systems and typically involves the endosomal sorting complex required for transport (ESCRT) or related pathways.[24] Vesicles are formed within multivesicular bodies, which subsequently fuse with the plasma membrane to release their contents—including lipids, proteins, and microRNAs—into the extracellular environment.[25] These cargo molecules play important roles in intercellular communication.

In contrast, PDNVs are not produced through a regulated biological process. Instead, they are generated artificially by mechanically or chemically disrupting plant tissues. During disruption, membrane fragments can re-seal into vesicle-like nanoparticles, and the resulting PDNV preparations therefore comprise a heterogeneous mixture of secreted EVs, intracellular vesicles/organelles, and reassembled membrane-derived particles.[26] As such, PDNVs represent a composite of intra- and extracellular materials, many of which are not naturally present in the extracellular space.[27]

Pretreatment Strategies: Gentle or Disruptive

The pretreatment strategies for PEVs and PDNVs vary markedly. PEVs are typically collected from apoplastic washing fluid (AWF) using standardized, low-impact techniques such as vacuum infiltration.[28] This method preserves cell wall integrity and yields vesicles with uniform morphology and consistent protein markers, including Tetraspanin 8 (TET8) and Heat Shock 70 kDa Protein (HSP70).[29]

Recent advances have improved the efficiency of PEV isolation, including the 3D-printed filtration tube[30] (Figure 1A), incorporating enzymatic digestion[31] (Figure 1B), and stem incision method across various plant species.[32] In plant tissue cultures, dedifferentiated cells with minimal cell wall specialization can secrete PEVs directly into the extracellular medium (Figure 1C). These approaches improve both the scalability and purity of the resulting vesicles (Figure 1D). Successful isolation has been demonstrated in cultured tomato,[33] tobacco,[34] seaweed,[35] and ginseng,[36] with the vesicles exhibiting consistent morphology and marker expression comparable to those obtained through traditional methods. Table 1 summarizes the core principles, advantages, and limitations of these PEVs pretreatment strategies.

In contrast, PDNVs are primarily obtained through mechanical disruption techniques, such as grinding[41] (Figure 1E), blending[42] (Figure 1F), and extrusion[43] (Figure 1G), which rupture plant cells. These methods release not only vesicles but also intracellular contents, non-vesicular particles, and cell wall debris (Figure 1H). In polysaccharide-rich plants such as citrus and aloe, the release of pectins and cellulose further complicates purification.[44] Moreover, variations in buffer composition, enzymatic activity,[27] applied shear forces,[45] and filtration protocols[46] contribute to the significant heterogeneity observed across PDNVs-related studies.

Physical Characteristics and Molecular Cargo

The pretreatment strategies of vesicles strongly influence the physical and molecular characteristics of PDNVs and PEVs (Table 2). PDNVs tend to be larger, more lipid-rich, and carry more RNA, but they also exhibit greater compositional complexity, likely due to contamination from cellular debris. In contrast, PEVs are generally smaller, more protein-enriched, and exhibit more consistent molecular profiles.

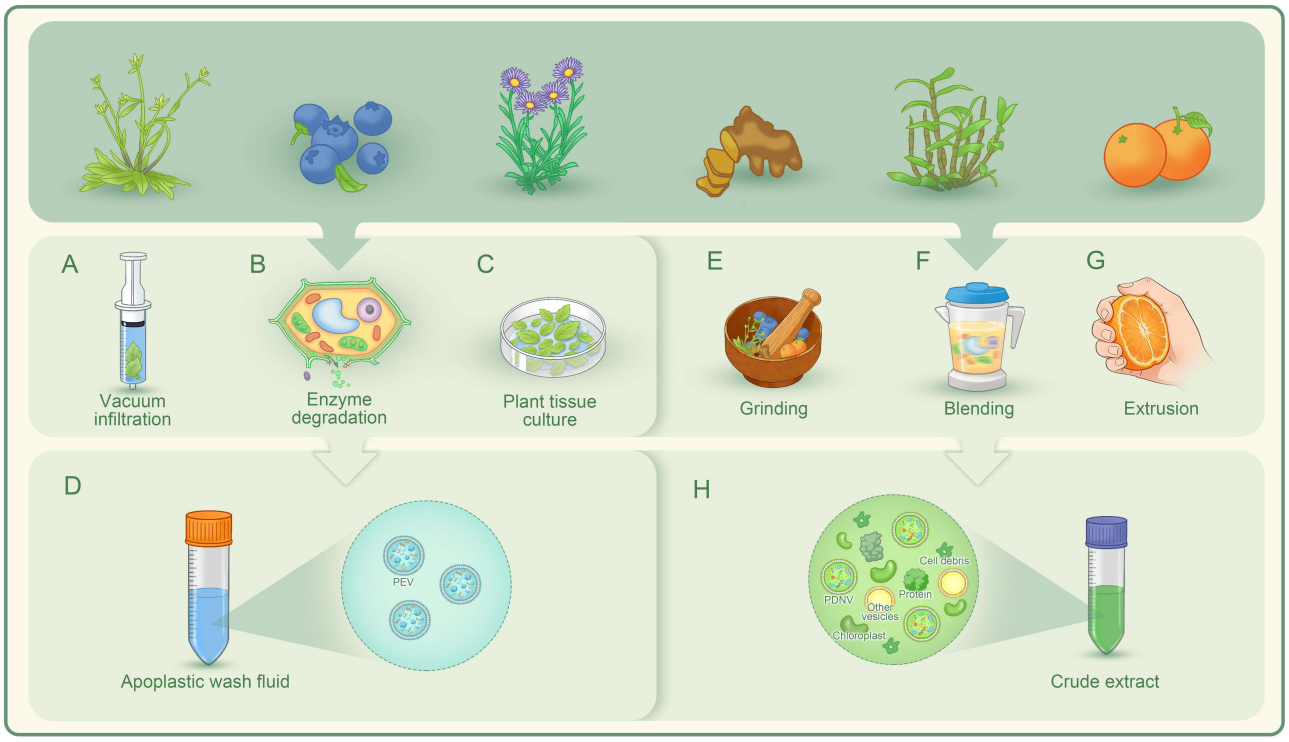


Figure 1 Representation of PEVs and PDNVs pretreatment strategies. Common pretreatment strategies for PEVs are vacuum filtration (**A**), enzymatic digestion (**B**) and plant tissue culture (**C**) to isolation of PEVs from apoplastic washing fluid (**D**). Common pretreatment strategies for PDNVs are tissue grinding (**E**), blending (**F**) and extrusion(**G**) to isolation of PDNVs from crude extract (H).

Particle Size

PEVs isolated from *Arabidopsis thaliana* using vacuum infiltration–centrifugation typically range from 60 to 200 nanometers. In contrast, PDNVs obtained through tissue homogenization display a broader size distribution, generally

Table 1 Sample Pretreatment Strategies for PEV Isolation

Pretreatment Methods	Principle	Source	Advantages	Disadvantages	References
Vacuum infiltration -centrifugation	Collection of apoplastic fluid	Plant leaves, seeds	1. High purity 2. Maintains cellular integrity	1. Low yield 2. Limited to leaves and seeds	[15,37]
3D-printed inner filters	Collection of apoplastic fluid	Fruit	1. High purity 2. Maintains cellular integrity	1. Low yield 2. Potential risk of cellular contamination	[30]
Stem incision	Collection of apoplastic fluid	Plant stems	1. Simple and rapid 2. Maintains cellular integrity	1. Restricted to stem-rich species	[32,38]
Enzymatic digestion	Digesting cell walls to release PEVs	Plant leaves, roots	1. High yield 2. No need for specialized instruments 3. Maintains cellular integrity	1. Time-consuming 2. Prolonged processing may affect vesicle integrity	[31,39]
Plant tissue culture	Plant cells secrete PEVs	Callus or suspension cultures	1. High yield 2. Broad plant applicability 3. Adjustable culture conditions 4. Standardize production potential	1. Long cultivation period	[27,29,33,34,39,40]

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Table 2 Differences Between PDNVs and PEVs in Physical Characteristics and Cargo Composition

Source	Pretreatment Method		Particle Size (nm)	Zeta Potential (mV)	Yield	Protein	Lipid	RNA	References
Morinda officinalis	Tissue disruption		50-120	N/A	(1.41 ± 0.52) ×10^9 particles/g	322.2 ± 10.51 μg/g	87.62%	More	[31]
	Enzymatic digestion		50-80	N/A	(4.35 ± 0.74) ×10^9 particles/g	423.8 ± 17.45 μg/g	66.17%	Less	
Arabidopsis	Tissue disruption		100-300	−26.3 ± 3.9	7.2 × 10^{10} particles/g	55.5 μg/g	N/A	N/A	[28]
	Vacuum infiltration		60-200	−28.7 ± 1.1	1.0 × 10^8 particles/g	130 ng/g	N/A	N/A	
Bok choy	Tissue disruption		N/A	N/A	6.31 × 10^9 particles/g	12.3 μg/g	N/A	N/A	[28]
	Vacuum infiltration		N/A	N/A	4.75 × 10^7 particles/g	58.2 ng/g	N/A	N/A	
Gai lan	Tissue disruption		N/A	N/A	2.42 × 10^{10} particles/g	28.6 μg/g	N/A	N/A	[28]
	Vacuum infiltration		N/A	N/A	7.06 × 10^7 particles/g	39.6 ng/g	N/A	N/A	
Cabbage	Tissue disruption		N/A	N/A	2.28 × 10^{10} particles/g	62.0 μg/g	N/A	N/A	[28]
	Vacuum infiltration		N/A	N/A	2.08 × 10^8 particles/g	238.1 ng/g	N/A	N/A	
Tobacco	Tissue disruption		209.3 ± 10.85	N/A	1.71 × 10^9 ± 3.46 × 10^8 particles/mL	96.19–506.18 μg/mL	N/A	N/A	[34]
	Plant tissue culture		175.5 ± 14.45	N/A	4.69 × 10^9 ± 1.06×10^9 particles/mL	713.6–1147.06 μg/mL	N/A	N/A	
Ginseng	Tissue disruption	DUC	29-499	−20.61	5.62 × 10^{11} particles/g	N/A	N/A	N/A	[47]
		EQ	42-389	−28.88	6.45×10^{11} particles/g	N/A	N/A	N/A	
		DUC+EQ	43-244	−29.54	1.56×10^{11} particles/g	N/A	N/A	N/A	
Catharanthus roseus	Plant tissue culture		72 ± 25.95	N/A	N/A	N/A	N/A	N/A	[36]
	Enzymatic digestion		75.51 ± 10.19	−21.8	0.80 ± 0.34 mg/g	N/A	N/A	N/A	[39]
	Plant cell culture	CrDDENs	77.83 ± 10.53	−21.2	2.58 ± 0.47	N/A	N/A	N/A	
		CrCDENs	65.72 ± 13.79	−31.2	2.73 ± 0.84	N/A	N/A	N/A	

Abbreviations: DUC, Ultracentrifugation; **EQ**, ExoQuick; **CrDDENs**, *Catharanthus roseus* Dedifferentiated cells-derived Exosome-like Nanovesicles; **CrCDENs**, *Catharanthus roseus* Cambial meristematic cells-derived Exosome-like Nanovesicles.

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between 100 and 300 nanometers. A similar trend is observed in *Morinda officinalis*, where enzymatically extracted extracellular vesicles from *Morinda officinalis* (MOEVs) range from 50–80 nm, whereas *Morinda officinalis* -derived nanovesicles (MONVs) obtained by tissue homogenization range from 50–120 nm.[31] PEVs isolated from tobacco and ginseng callus cultures also exhibit smaller average diameters (175.5 ± 14.45 nm and 72 ± 25.95 nm, respectively) compared to their PDNV counterparts (209.3 ± 10.85 nm and 92.04 ± 4.85 nm).[34,36] The consistent size differences observed between PEVs and PDNVs across various plant species suggest that pretreatment strategies play a critical role in determining vesicle size. These variations likely reflect fundamental differences in vesicle origin and structural integrity.

Vesicle Yield

Yield comparisons also demonstrate notable discrepancies. Liu et al reported that PEVs from *Arabidopsis* isolated via vacuum infiltration yielded approximately 1.0×10^8 particles per gram of tissue, whereas tissue homogenization produced about 7.2×10^{10} particles per gram.[28] However, milder methods such as enzymatic digestion or cell culture can produce PEVs in yields up to threefold higher than those of PDNVs.[31,34] These findings suggest that although vacuum-based methods offer higher purity, physical barriers such as the plant cell wall may limit vesicle recovery.

Cargo Composition

Proteomic analyses underscore distinct compositional differences. PDNVs from *Arabidopsis* contain approximately 1,438 unique proteins—nearly twice the 787 proteins identified in corresponding PEVs.[28] Despite this numerical disparity, PEVs generally exhibit a higher protein concentration per vesicle.[28,31,34]

In terms of RNA cargo, MONVs contain higher levels of microRNAs and other non-coding RNAs compared to MOEVs, likely due to the non-specific inclusion of cytoplasmic RNA during mechanical disruption.[31] Lipidomic profiling also reveals distinct patterns: MOEVs are enriched in phosphatidylcholine (46.40%), phosphatidylethanolamine (29.06%), and phosphatidylserine (7.71%), whereas MONVs contain lower levels of phosphatidylcholine (29.18%) and phosphatidylethanolamine (14.86%) but a higher proportion of phosphatidylinositol (8.53%). Furthermore, the relative proportion of lipids and lipid-like molecules is greater in MOEVs (87.62%) than in MONVs (66.17%).[31] Metabolomic analyses further differentiate MOEVs and MONVs. Compared to MONVs, MOEVs display 114 downregulated and 68 upregulated metabolites. KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis suggests that these differences may influence biological processes, particularly those related to plant growth and development.[31] These findings highlight the distinct molecular compositions of MONVs and MOEVs, suggesting differences in their biogenesis and potential functional roles in intercellular communication.

Functional and Therapeutic Implications

The physical and molecular distinctions between PDNVs and PEVs have significant implications for their biological functions and therapeutic applications. These differences directly influence cellular interactions, uptake mechanisms, and downstream effects on cellular processes.

In terms of cellular uptake, PEVs generally exhibit greater efficiency. For instance, PEVs derived from *Arabidopsis* were internalized by cancer cells at rates nearly three times higher than comparable PDNVs, with similar trends observed in endothelial cells.[28] Moreover, MOEVs demonstrated superior uptake compared to MONVs under both low and high concentration conditions. At low concentrations, uptake rates were 11.2% for MOEVs versus 4.78% for MONVs; at higher concentrations, uptake increased to 75.7% and 55.4%, respectively.[31]

Despite lower cellular uptake, PDNVs may offer advantages in tissue-specific targeting. In vivo imaging studies revealed that MONVs accumulated more extensively in bone tissue than MOEVs at 12, 24, and 48 hours post-administration.[31] These findings suggest that, while PEVs are more efficiently internalized at the cellular level, PDNVs may achieve more effective biodistribution in certain tissues.

Functional outcomes further distinguish the two vesicle types. Both ginseng-derived PEVs and PDNVs have demonstrated the ability to suppress melanin production; however, PEVs were more effective in reducing markers of cellular senescence, such as senescence-associated β-galactosidase.[36] These observations indicate that, although some functional overlap exists, PEVs and PDNVs possess distinct biological activities.

Taken together, these differences underscore the need for clearer classification criteria. PEVs and PDNVs are not interchangeable, as each exhibits unique functional profiles and therapeutic potentials. Rigorous separation and characterization of these vesicle populations are essential to advance both fundamental research and translational applications.

In summary, PDNVs differ fundamentally from PEVs in their pretreatment strategies, leading to significantly greater heterogeneity among PDNVs. To enhance reproducibility and facilitate clinical translation, future research on PDNVs must address these limitations directly. Key priorities include adopting standardized procedures and improving purification protocols.[42] The following section provides a detailed overview of mainstream PDNVs isolation techniques. It examines the underlying principles of each method, highlights their advantages, and discusses associated limitations.

Isolation — The Unsolved Engineering Challenge

A major obstacle to the reliable application of PDNVs lies in their isolation and purification processes. Table 3 provides an overview of purification strategies and summarizes their distinguishing features.

Density-Based Isolation Strategies for PDNVs

Among density-based isolation techniques for PDNVs, DUC and DGC remain classical methods, each offering distinct yet complementary advantages.

Differential Ultracentrifugation (DUC)

DUC is the most commonly employed technique for isolating PDNVs and is widely regarded as the "gold standard" for extracellular vesicle extraction from animal cells.[48] This method separates particles based on their sedimentation rates in response to increasing centrifugal forces. Typically, low-speed centrifugation (<3,000 ×g) is used to eliminate large plant debris and fibers, followed by medium-speed centrifugation (<15,000 ×g) to remove residual cell fragments. Finally, ultrahigh-speed centrifugation (>100,000 ×g) is applied to pellet PDNVs.[49]

To enhance purity, many protocols incorporate sequential filtration steps using 0.45 μm or 0.22 μm membranes to exclude larger residual particles, thereby enriching for vesicles within a size range of approximately 30–200 nm.[16,60] However, in the absence of standardized protocols, critical parameters such as g-force, spin duration, and step sequences vary considerably across published studies.[48]

Despite its widespread use, DUC has several notable limitations. It requires access to specialized ultracentrifugation equipment and involves prolonged processing times. Extended exposure to excessive centrifugal force may cause vesicle deformation or aggregation, potentially compromising structural integrity and biological functionality.[61,62] In addition, DUC frequently co-isolates non-specific proteins and contaminants, which can interfere with downstream functional analyses.[61]

To overcome these challenges, hybrid approaches combining DUC with DGC are increasingly adopted. This integrated methodology improves vesicle purity and isolation efficiency, enabling the recovery of more biologically relevant PDNVs populations.[63]

Density Gradient Centrifugation (DGC)

DGC separates PDNVs based on their buoyant density by layering continuous density gradients—commonly sucrose solutions at concentrations of 8%, 15%, 30%, 45%, and 60%—within centrifuge tubes.[64] During ultracentrifugation, particles migrate to the position where their density equals that of the surrounding medium, enabling precise separation.[65] PDNVs typically accumulate between the 30% and 45% sucrose layers, whereas contaminants such as soluble proteins and cellular debris are distributed across other density fractions.[28,66]

Compared to DUC, DGC offers superior vesicle purity, particularly by more effectively eliminating co-isolated proteins and other contaminants. Notably, DGC is currently the only reported technique capable of efficiently removing viral contaminants from PDNV preparations,[67] further enhancing sample integrity.

Despite its advantages, DGC remains labor-intensive, time-consuming, and cost-prohibitive, which limits its feasibility for large-scale or industrial PDNV production.[68]

Table 3 Principles, Advantages, and Disadvantages of Different Isolation Techniques for PDNVs

Isolation Methods	Principle	Advantages	Disadvantages	References
DUC	Particle size and density	(1) Low cost (2) Large-scale sample processing	(1)Time-consuming and labor-intensive (2) Requires specialized equipment (3) Risk of vesicle rupture or aggregation (4) Low recovery	[48,49]
DGC	Particle density	(1) High purity and recovery (2) Removal of viral contaminants	(1) Time-consuming and labor-intensive (2) Requires specialized equipment	[48,49]
UF	Particle size	(1) Sample concentration (2) Fast and convenient (3) Large-scale sample processing	(1) Membrane clogging may occur (2) Low purity	[50,51]
SEC	Particle size	(1) High purity (2) Simple operation	(1) Limited sample loading capacity	[52]
ELD	Particle size and electric field	(1) Efficient and fast (2) No complex equipment required	(1) Requires manual operation	[53]
PEG precipitation	Vesicle hydrophobicity	(1) Low cost (2) Large-scale sample processing (3) No specialized equipment required	(1) Low purity (2) High PEG concentrations may affect vesicle bioactivity	[54,55]
HIC	Vesicle hydrophobicity	(1) Rapid (<15 min) (2) High yield (3) Low cost (4) No specialized equipment required	(1) Potential impact of elution additives on vesicles (2) Limited loading capacity, low throughput	[56]
ATPS	Vesicle hydrophobicity	(1) Low cost and high yield (2) Fast and convenient (3) No specialized equipment required	(1) Residual dextran may affect downstream analysis	[57,58]
Immunoaffinity capture	Antigen-antibody specific binding	(1) High specificity and purity	(1) Low yield and high cost (2) Lack of specific magnetic beads and antibodies	[37,59]

Abbreviations: DUC, Differential Ultracentrifugation; **DGC**, Density Gradient Centrifugation; **UF**, Ultrafiltration; **SEC**, Size-Exclusion Chromatography; **ELD**, Electrophoresis-linked dialysis; **PEG precipitation**, Polymer-based precipitation; **HIC**, Hydrophobic Interaction Chromatography; **ATPS**, Aqueous Two-Phase System.

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Size-Based Isolation Strategies for PDNVs

PDNVs typically range from 30 to 1000 nm in diameter,[69] rendering size-exclusion principles highly applicable for their isolation. Techniques such as UF, SEC, and ELD leverage these size differences to enable efficient separation from soluble or particulate impurities.

Ultrafiltration (UF)

UF employs membranes with defined pore sizes to selectively separate PDNVs from smaller contaminants.[50] It enables sample processing within 30–40 minutes using standard laboratory equipment, making the method both time-efficient and cost-effective.[70,71] UF has been successfully applied for the extraction and purification of PDNVs from various plant sources, including kudzu root,[70] *Dendrobium officinale*,[71] *Dendropanax morbifera*,[38] *dandelion*,[72] and *lemon*.[73] Compared to DUC, UF offers the dual advantages of sample concentration and impurity removal. Moreover, it is simpler to operate and more suitable for large-scale production.[74]

However, UF has limited resolution in separating particles of similar size to PDNVs and is prone to membrane fouling by plant-derived proteins, which can compromise vesicle purity.[75] For instance, comparative studies of DUC, UF, and polymer-based precipitation for isolating *Arabidopsis* extracellular vesicles found that while UF preserved vesicle morphology effectively, it introduced the highest levels of protein contamination.[76]

Tangential flow filtration (TFF), a refined version of UF, addresses this issue. By directing fluid tangentially across the membrane surface, TFF reduces protein buildup and membrane clogging.[77] Nevertheless, TFF requires specialized equipment, which increases both procedural complexity and operational cost.

Electrophoresis-Linked Dialysis (ELD)

ELD developed by Yang et al, integrates electric-field-driven migration with dialysis to enable efficient isolation of PDNVs.[53] Although this technique is promising, its adoption remains limited. In the ELD system, an electric field facilitates the migration of small molecules—such as nucleic acids and proteins—across a 300 kDa dialysis membrane, while larger PDNVs are retained within the dialysis bag. To mitigate membrane fouling and vesicle aggregation, the system applies polarity-reversal pulses every 30 minutes.[43] ELD yields PDNVs with particle sizes and concentrations comparable to those obtained through DUC. Subsequent studies have successfully employed ELD to isolate vesicles from bitter melon, underscoring its versatility and potential for broader application.[53]

Size-Exclusion Chromatography (SEC)

SEC isolates PDNVs based on their hydrodynamic radius using columns packed with porous stationary phases. Larger PDNVs are excluded from entering the pores and elute earlier, whereas smaller proteins, RNA, and other contaminants penetrate the pores and are retained longer within the gel matrix, enabling effective separation.[52] SEC has been effectively employed to purify PDNVs from pomegranate[78] and *Citrus limon*,[79] preserving intact membrane structure and critical bioactive components.

Compared with DUC, SEC provides advantages including shorter processing time, standardized protocols, moderate equipment requirements, and high reproducibility. However, limitations remain, including complex sample preparation, low sample throughput, and reduced efficacy in removing contaminants of similar size to PDNVs.

Hydrophobic Characteristics-Based Isolation Strategies for PDNVs

The phospholipid bilayer of PDNVs confers surface hydrophobicity, which serves as a useful property for their separation from plant extracts.[80] Current hydrophobicity-based isolation methods include HIC, polymer-based precipitation, and ATPS. These techniques demonstrate strong potential for efficient and scalable PDNV purification.

Hydrophobic Interaction Chromatography (HIC)

HIC is a chromatographic technique that separates molecules based on differences in surface hydrophobicity. It leverages the intrinsic hydrophobic characteristics of EVs, using capillary channel polymer (C-CP) fibers as the stationary phase.[81] Under high-salt conditions, vesicles bind to the column; as the salt concentration is gradually reduced, vesicles are eluted according to their degree of surface hydrophobicity.[82]

HIC has been successfully applied to purify EVs from various biological fluids, including urine,[83] plasma,[84] and cell culture media.[82,83] Jackson et al were the first to apply HIC for the isolation and purification of PDNVs. In their study, HIC enabled the enrichment of PDNVs at high concentrations (>1 × 10^{10} particles/mL) from as little as 100 μL of plant juice, with a total processing time of approximately 15 minutes. The isolated vesicles exhibited an average size of 189 nm and retained intact morphology, indicating high recovery efficiency.[56] PDNVs were successfully isolated from different fruits and vegetables, demonstrating the method's broad applicability and versatility.

However, protein contaminant removal efficiency varied significantly by plant source, ranging from 48% to 95%. Additionally, the effects of HIC elution buffers on vesicle integrity remain unclear. Further investigation and methodological standardization are needed to optimize HIC for broader use in PDNVs applications.

Polymer-Based Precipitation

Polymer-based precipitation is a widely employed method for PDNVs. This technique relies on hydrophilic polymers like polyethylene glycol (PEG), which compete for solvent molecules, reducing vesicle solubility and inducing PDNV aggregation and precipitation.[43] Also termed PEG precipitation, it offers simplicity, cost-effectiveness, minimal equipment requirements, and structural preservation – making it prevalent in extracellular vesicle purification.[85]

Nevertheless, this approach has significant limitations. The process typically necessitates prolonged incubation (often overnight), rendering it unsuitable for rapid isolation.[86] It also lacks specificity, frequently co-precipitating contaminants including soluble proteins, free nucleic acids, and viral particles, thereby compromising purity and downstream analyses.[87] Furthermore, PEG concentration impacts vesicle bioactivity. For instance, high PEG6000 concentrations (12%–15%) significantly reduced total polyphenol content and antioxidant capacity in ginger-derived nanovesicles.[54] While commercially available kits such as ExoQuick offer convenience, they incur high costs and yield vesicles of relatively low purity compared to ultracentrifugation or density gradients.[88] These drawbacks constrain their utility for large-scale production or clinical translation.

Aqueous Two-Phase Systems (ATPS)

ATPS offer a mild yet efficient separation approach, typically comprising two immiscible hydrophilic polymers such as PEG and dextran (DEX).[89] Separation exploits differences in PDNV surface hydrophobicity and physicochemical properties, driving preferential partitioning into the DEX-rich phase while retaining soluble contaminants in the PEG-rich phase.[90]

Compared to DUC, ATPS achieves several-fold higher vesicle recovery and eliminates >95% of protein contaminants.[91] Kırbaş et al further optimized ATPS protocols for diverse plant-derived samples, enhancing removal of organic acids and proteins.[57] This method provides distinct advantages over conventional techniques, including simplicity, reduced processing time, minimal equipment dependence, and scalability.

Nevertheless, persistent limitations constrain wider adoption. Residual DEX increases sample viscosity and compromises downstream applications (eg., Western blotting, RNA extraction, cellular assays) through non-specific biopolymer interaction.[58] Consequently, optimizing system composition and extensive purification to balance vesicle recovery with sample purity remains critical for advancing ATPS technology.

Surface-Specific Proteins-Based Separation Strategies for PDNVs

Compared to physicochemical property-based isolation strategies, surface marker-targeted approaches provide enhanced specificity and resolution.[92] Immunoaffinity capture exemplifies this paradigm, leveraging antibodies against vesicular surface proteins for precise isolation.

Immunoaffinity Capture

Immunoaffinity capture achieves high-precision isolation of PEVs by targeting specific membrane surface proteins, widely recognized as the most accurate method for isolating defined EVs subpopulations.[37] This technique exploits antibody-antigen interactions with vesicular surface markers to selectively isolate vesicles bearing unique protein signatures.[93] Notably, TET8 —a tetraspanin identified in *Arabidopsis thaliana*—emerges as a conserved PEVs marker. Building on this, He, B. et al

developed a TET8-targeted immunocapture system using magnetic beads conjugated to antibodies against its extracellular loop 2 (EC2) extracellular loop, facilitating selective isolation of TET8-positive PEVs.[59]

Despite unparalleled specificity, immunoaffinity capture faces significant hurdles in PDNVs applications. The scarcity of conserved, universally recognized PEVs surface markers[88] restricts antibody-based strategies, while scalability remains constrained by high costs, low yields, and batch-to-batch antibody variability.

Each isolation method presents trade-offs among key parameters such as purity, yield, structural integrity, reproducibility, and scalability. No single technique demonstrates optimal performance across all criteria. Moreover, most PDNVs lack comprehensive vesicle characterization, and few have conducted direct comparative analyses of isolation approaches. Additional challenges arise from batch-to-batch variability and differences among plant species, further complicating efforts toward standardization. These limitations are particularly concerning given the ongoing ambiguity surrounding the fundamental definition of PDNVs.

Rebuilding the Foundation — Towards Quality, and Scale

Optimizing Pretreatment Conditions

Strategic optimization can substantially enhance vesicle isolation efficiency and reproducibility. Figure 2A–C provides a visual summary of pretreatment strategies designed to enhance the yield and purity of PEVs and PDNVs.

Tissue and Species Selection

Different plant species and tissue types exhibit considerable variability in vesicle yield, morphology, and molecular composition[94] (Figure 2A). To enhance vesicle purity, selective removal of plant structures known to contribute non-vesicular contaminants can be beneficial. For example, in the extraction of PEVs, removing the petioles of *Arabidopsis thaliana* significantly reduced contamination from Rubisco protein and xylem sap in the recovered apoplastic fluid.[37]

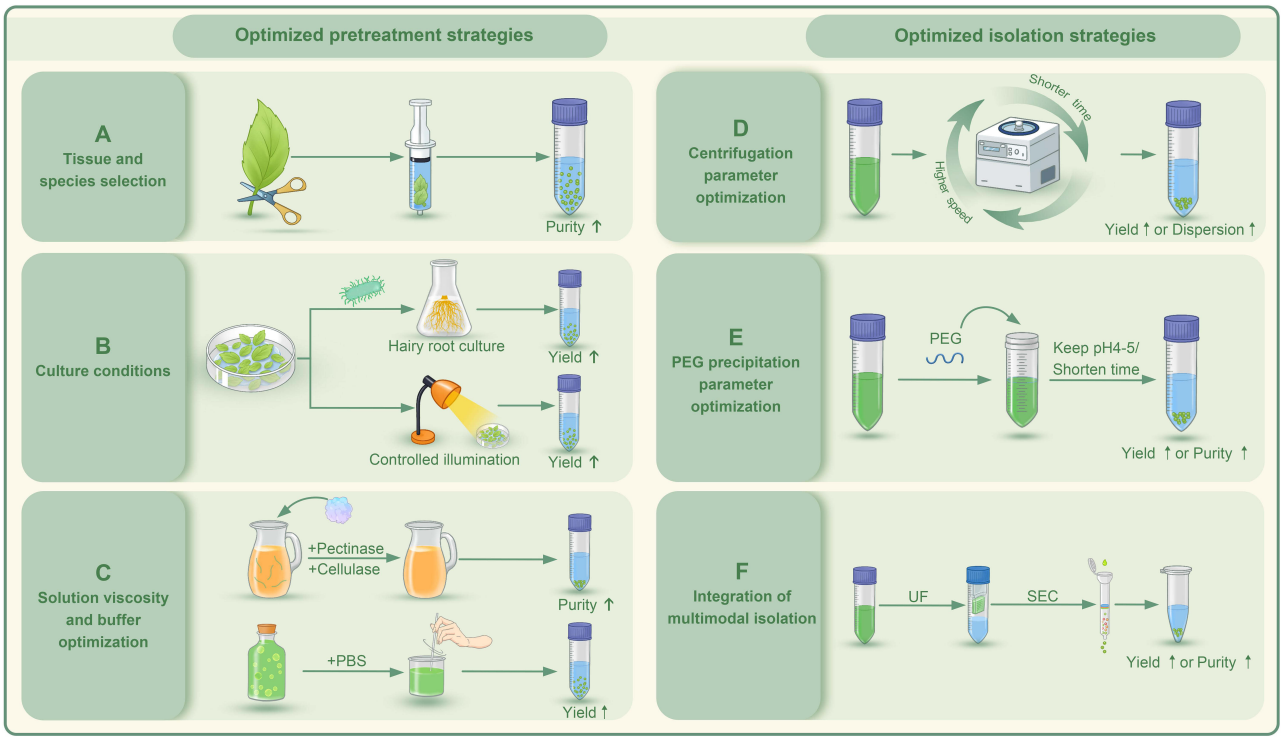

Figure 2 Representation of optimized pretreatment and isolation strategies for PEVs and PDNVs. Optimization of pretreatment strategies such as selection of different parts of the plant or different species (**A**), optimization of culture conditions (**B**), and optimization of solubilization (**C**) can improve the yield or purity of PEVs or PDNVs. Optimization of separation strategies such as centrifugation conditions (**D**), PEG conditions (**E**), and integration of multimodal strategies (**F**) can increase the yield or purity of PEVs or PDNVs.

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Similarly, PDNVs isolated from *Catharanthus roseus* leaves displayed uniform, exosome-like morphology, whereas vesicles from stems and flowers exhibited distinct structural characteristics, differing notably from those derived from leaves.[39]

Culture Conditions

Biological stimulation also offers promising strategies to increase vesicle yield[59] (Figure 2B). Fungal infection by *Botrytis cinerea*, for instance, enhances vesicle secretion in *Arabidopsis*.[37] Likewise, *Agrobacterium rhizogenes*-induced hairy root cultures provide a scalable, hormone-independent platform for vesicle production.[40] Tissue culture systems further support high-yield vesicle production, with 50 L bioreactors achieving gram-scale output—nearly threefold higher than enzymatic digestion methods.[39] Moreover, culture condition optimization has demonstrated efficacy in boosting vesicle secretion; for example, magenta light emitting diode (LED) illumination increased vesicle yield from *Leontopodium nivale* callus by 2.4-fold compared to dark conditions, while also enhancing secondary metabolite and protein synthesis.[95]

Solution Viscosity and Buffer Optimization

Solution viscosity is another critical factor affecting isolation efficiency and overall recovery[44] (Figure 2C). During tissue disruption, the release of polysaccharides such as pectin and cellulose increases viscosity and reduces vesicle purity.[48] To address this, enzymatic degradation using pectinase and cellulase has been proposed to reduce polymer accumulation.[96] Additional purification strategies include washing PDNVs with Tris-HCl buffer, which effectively removes macromolecular impurities.[97] Similarly, diluting aloe vera juice with phosphate-buffered saline (PBS) significantly improved recovery of aloe-derived nanovesicles (ADNVs).[98] Building on these results, treatment of aloe tissues with a hypotonic ICG (indocyanine green)-containing solution enhanced not only ICG encapsulation but also vesicle secretion, structural integrity, and mechanical stability, ultimately improving tumor penetration capacity.[99]

Use of Protective Agents

The use of protective agents during isolation can further reduce vesicle loss and aggregation (Figure 2D and E). For example, sucrose density gradient buffers containing dual layers (68% and 27% sucrose) have been shown to minimize aggregation and rupture of ginseng-derived PDNVs during centrifugation, significantly improving recovery.[100] This method has also been successfully applied to *Panax notoginseng*[101] and *citrus*.[102] Additionally, the use of a 60% iodixanol gradient was effective in protecting ginger-derived PDNVs from damage during centrifugation.[103]

Precise Isolation Parameters

Centrifugation parameters critically determine vesicle yield (Figure 2D). DUC remains the primary PDNV isolation method, typically requiring >100,000 × g forces and durations exceeding 1 hour.[104] In aloe-derived PDNVs, ultracentrifugation at 100,000 × g for 60 min produced obvious agglomerations on TEM, with a broadened size range (≈50–500 nm) and markedly increased dispersity; DLS further showed a maximum size of 553.4 nm and PDI up to 0.59 at 60 min. In contrast, under the same g-force, shortening the duration improved monodispersity (PDI 0.21 at 20 min; 0.14 at 10 min), meeting the commonly accepted threshold for lipid vesicles in drug delivery (PDI ≤ 0.3).[98] Notably, Zeng et al selected 20 min as a practical compromise between yield (total protein as a proxy) and vesicle quality.[98] Similarly, Chen et al achieved uniform, well-dispersed cucumber PDNVs by optimizing ultracentrifugation duration.[105] Elevated centrifugal forces also enhance recovery. Huang et al demonstrated substantially higher vesicle yields at 100,000 × g versus 40,000 × g for *Arabidopsis* extracellular vesicles, with significant vesicle retention in supernatants at lower forces.[37]

Supplementary parameter optimizations further improve outcomes (Figure 2E). Shortening aloe PDNV PEG precipitation from 24 to 16 hours enhanced purity without compromising yield.[106] Similarly, mildly acidic conditions (pH=4–5) during ginger PDNVs PEG precipitation increased yield 4–5 fold while preserving polyphenol content and bioactivity.[55] Collectively, precise calibration of isolation parameters significantly boosts PDNVs yield and purity, establishing foundations for scalable production.

Integrate Multi-Modal Isolation Technologies

In addition to optimizing individual methods, integrating complementary isolation techniques significantly enhances vesicle purity (Figure 2F). Given the heterogeneity of plant matrices, single-method approaches rarely achieve both high enrichment and high-resolution purification. Consequently, synergistic workflows combining enrichment and refinement steps are increasingly adopted.

Schatz D et al successfully separated *diatom* extracellular vesicles from virions using TFF with DGC.[35] SEC visibly decolorized blueberry juice—from purple to transparent—demonstrating exceptional purification capacity[107] (Figure 3A). However, SEC's limited loading capacity constrains scalability.[79] To address this, researchers combine SEC with DUC or UF for efficient enrichment and refinement. For instance, Bokka et al used DUC for initial enrichment of tomato-derived nanovesicles followed by DGC and SEC, with DUC+SEC showing superior impurity removal.[108] Similarly, You JY et al demonstrated that UF+SEC outperformed DUC and PEG precipitation for cabbage nanovesicles, yielding vesicles with more uniform size distribution, higher yield per unit, and enhanced purity[51] (Figure 3B and C). This hybrid approach also efficiently isolated carrot PDNVs.[109] Jang et al further achieved 83.3% relative purity —1.5–2.4 times higher than individual methods—by combining ultracentrifugation (DUC/DGC) with ExoQuick™.[47]

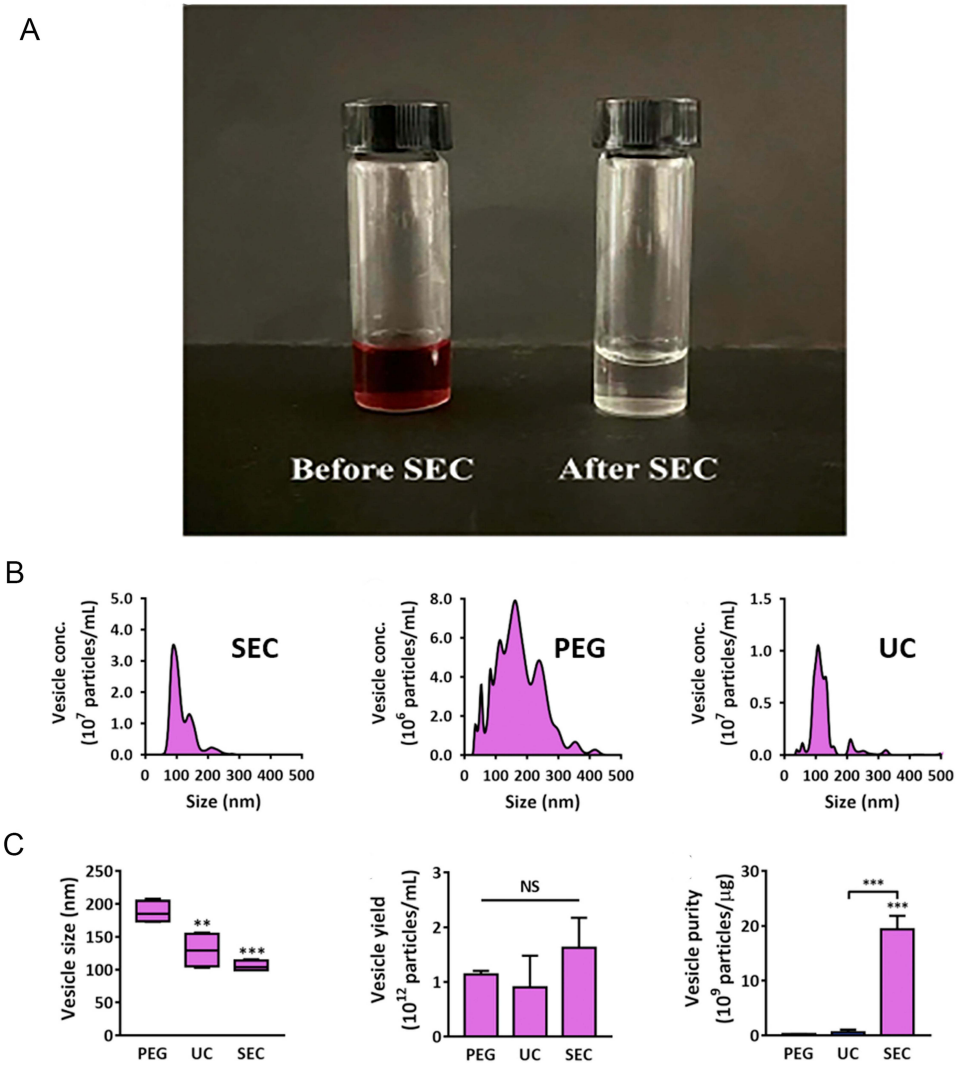

Figure 3 Representation of integrate multi-modal isolation strategies. (**A**) Photographs of centrifuged blueberry juice before and after SEC.[107] (**B**) Comparison of NTA size distribution profiles of Rabex isolated by SEC+UF, PEG precipitation, and UC.[51] (**C**) Comparison of average particle size, nanovesicle yield, and purity of Rabex across different isolation methods by SEC+UF, PEG precipitation, and UC.[51] Statistical significance: **p < 0.01, ***p < 0.001; NS, not significant.[51]

Abbreviations: NTA, nanoparticle tracking analysis; SEC, size-exclusion chromatography; PEG, PEG-based precipitation; UC, ultracentrifugation; UF, ultrafiltration.

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Collectively, these studies underscore that synergistic strategies integrating enrichment and purification markedly improve PDNV purity. Note that purity gains often correlate with reduced recovery, particularly in multi-step protocols.[47] Researchers should therefore balance purity, yield, and intended applications—whether compositional profiling, functional assays, or clinical translation—by selecting context-appropriate isolation schemes.

Implement Purity Indexing and Bioactivity Normalization

To Enable Cross-Study Comparability, We Recommend Two Standardized Metrics

The Particles/Protein Ratio (Particles/μg Protein)

The particles/protein ratio (particles/μg protein) is a metric for evaluating the purity of extracellular vesicle preparations.[50] The ratio is calculated by dividing the total number of particles by the protein content (in micrograms), providing an indirect estimate of non-vesicular protein contamination. Protein concentration is typically determined using the bicinchoninic acid assay (BCA), while vesicle particle counts are measured via nanoparticle tracking analysis (NTA) or nano-Flow Cytometry (nFCM). A higher particles-to-protein ratio indicates a greater proportion of vesicles relative to free proteins, and thus, higher sample purity.

IC_{50} (Half Maximal Inhibitory Concentration) per 10^{10} Particles

The IC_{50} per 10^{10} particles is a metric for evaluating the biological potency of EVs, achieved by normalizing the IC_{50} value to a defined vesicle count.[110] This normalization minimizes variability due to differences in sample concentration, enabling more accurate comparisons across studies and vesicle types. Vesicle concentration is typically quantified using NTA, which measures the number of particles in suspension. The resulting IC_{50} value represents the number of vesicles required to elicit 50% of the maximal biological response. A lower IC_{50} per 10^{10} particles indicates greater potency per vesicle, reflecting enhanced biological activity and functional efficiency.

Collectively, these strategies establish a roadmap for consistent, scalable production of PDNVs. As a practical next step, future studies should report batch-to-batch variability, particles/protein ratios, and potency-normalized bioactivity in a harmonized manner. Provisional working targets may help guide early standardization efforts and these targets should be refined through interlaboratory validation.

Conclusion and Future Perspectives

PEVs and PDNVs represent a rapidly evolving frontier at the intersection of plant biology, nanotechnology, and therapeutic innovation. These vesicles, distinguished by their intrinsic biocompatibility and diverse molecular cargo, hold substantial promise as sustainable nanocarriers.

However, progress in this domain is impeded by persistent ambiguities in classification and nomenclature. Establishing a pragmatic, reporting-oriented classification and implementing standardized, assay-appropriate purity and potency reporting represent the most immediate needs to improve comparability and accelerate translation.

Future advances will depend on the integration of next-generation technologies. Genome editing, synthetic biology, and plant-based biomanufacturing platforms offer new avenues to enhance vesicle yield and engineer functional cargo profiles. Concurrently, transitioning from conventional ultracentrifugation to innovative isolation strategies—such as microfluidic systems[111] and affinity-based methods[112]—can improve vesicle purity, reproducibility, and scalability.

For successful clinical and commercial translation, the field must establish rigorous quality control and safety assessment frameworks. Comprehensive evaluations of immunogenicity, biodistribution, and toxicity, coupled with harmonized regulatory standards, are essential.[43] Initial safety data from edible plant sources (ginger, lemon, aloe) suggest low immunogenicity, but systematic toxicology studies across diverse PDNVs sources remain absent from literature.[64,73,99] If these foundations are established, selected PDNV formulations may become candidates for early-phase clinical testing in the coming years. Through coordinated, system-level efforts, PDNVs have the potential to become reliable, scalable platforms for next-generation therapeutics and sustainable healthcare solutions.

Abbreviations

ADNVs, aloe-derived nanovesicles; ATPS, aqueous two-phase systems; AWF, apoplastic washing fluid; BCA, bicinchoninic acid assay; C-CP, capillary channel polymer; DEX, dextran;DGC, density gradient centrifugation; DUC, differential ultracentrifugation; EC2, extracellular loop 2; ELD, electrophoresis-linked dialysis; Erα, estrogen receptor alpha; ESCRT, endosomal sorting complex required for transport; EVs, extracellular vesicles; HIC, hydrophobic interaction chromatography; HPDENs, *Hypericum perforatum*-derived exosomes-like nanovesicles; HSP70, Heat Shock 70 kDa Protein; IC_{50}, half maximal inhibitory concentration; ICG, indocyanine green; KEGG, Kyoto Encyclopedia of Genes and Genomes; LED, light emitting diode; MISEV, Minimal Information for Studies of Extracellular Vesicles; MOEVs, *Morinda officinalis* extracellular vesicles; MONVs, *Morinda officinalis*-derived nanovesicles; nFCM, nano-Flow Cytometry; NTA, nanoparticle tracking analysis; PDNVs, plant-derived nanovesicles; PEG, polyethylene glycol; PEVs, plant extracellular vesicles; RDNVs, *Rhizoma Drynariae*-derived nanovesicles; ROS, reactive oxygen species; SEC, size-exclusion chromatography; TET8, Tetraspanin 8; TFF, tangential flow filtration; UF, ultrafiltration.

Data Sharing Statement

Data sharing not applicable – no new data generated: Data sharing is not applicable to this article as no new data were created or analyzed in this research.

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. All authors have read and agreed to the published version of the manuscript.

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Disclosure

The authors report no conflicts of interest in this work.

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