Modified Plant-Derived Nanovesicles for Therapeutics Delivery

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Extracellular vesicles (EVs) are a highly heterogeneous population of membranous particles that are secreted by almost all types of cells across different domains of life, including plants. Studies on plant-derived nanovesicles (PDNVs) showed that they could modulate metabolic reactions of the recipient cells, affecting (patho)physiology with health benefits in a trans-kingdom manner. In addition to its bioactivity, PDNV has advantages over conventional nanocarriers, making its application promising for therapeutics delivery.

plant-derived nanovesicles extracellular vesicles exosomes

1. Introduction

Consumption of certain foods or their associated components is often linked to health benefits and disease risk reduction. Such bioactive compounds are generally derived from plants: plant-originated micronutrients, sterols, fibers, polyphenols, and other phytochemicals, which scientists have studied to understand their impact on health. Recently, expansion of extracellular vesicle (EV) research suggested that plant-derived nanovesicles (PDNVs) can be a new member of dietary components with biological impacts.

PDNVs carry a wide array of molecules, including biologically active metabolites, proteins, lipids, and nucleic acids, making them small but mighty vectors capable of modulating metabolic phenotypes in recipient cells. They have gained scientific interest as it became clear that the cell-to-cell communication mediated by the nanoparticle is possible between different species [1][2]. Multiple studies have shown the feasibility of utilizing PDNVs as a means to boost optimal health by complementing insufficient consumption of fruits and vegetables. Furthermore, the nano structure of PDNVs, that allows encapsulation of various types of molecules into the hydrophilic core and the surrounding lipid layer, makes them a natural nanocarrier. In this regard, clinical studies are currently ongoing to determine the effects of PDNVs on human health: whether plant exosomes can deliver curcumin to colon tumors and the normal colon effectively (NCT01294072); whether ginger-derived exosomes, alone or combined with curcumin, ameliorates symptoms of patients with inflammatory bowel disease (NCT04879810); whether grape exosomes the incidence of radiation and chemotherapy-induced oral mucositis (NCT01668849); and whether a natural supplement containing nanovesicles delivered from *Citrus Limon* (L.) juice reduces several cardiovascular risk factors (NCT04698447) [3].

2. Origin and Nomenclature of PDNVs

According to the guidelines published in 2018 by the International Society for Extracellular Vesicles (MISEV2018), the term “EV” can be used for “particles naturally released from cells that are delimited by a lipid bilayer and cannot replicate” [4]. EVs can be classified based on their subcellular origins: exosomes (less than 150 nm in diameter), microvesicles/microparicles/ectosomes (MVs) (100 nm^{-1} μm in diameter), and apoptotic bodies (1–5 μm in diameter) [5]. Exosomes are the smallest EV subtype generated by the inward budding of an endosomal membrane via the endosomal sorting complex required for transport. The resultant intraluminal vesicles are present inside the multivesicular body (MVB) before being secreted to extracellular space as exosomes when MVB fuses with the plasma membrane. On the other hand, MVs are generally larger than exosomes and originated from direct local outward budding of the plasma membrane. First, lipids and membrane-associated proteins form a cluster in plasma membrane microdomains. In parallel to exosomes, such microdomains recruit soluble components, including membrane proteins, cytosolic proteins, and RNA species [5][6]. This cluster promotes membrane budding and subsequent release. The flipping of phosphatidylserines between the leaflets of the budding membrane is unique to MV biogenesis [6]. Although the terms based on unique biogenesis are generally accepted in the field, specific markers of subcellular origins are not yet established, which often generates inaccurate assignment of EVs [4]. Therefore, physical characteristics, such as size and density, biochemical composition (exosomal protein markers), or descriptions of collecting conditions (e.g., hypoxia, apoptosis, etc.) are recommended as standards to subdivide EVs into groups [4].

Categorizing PDNVs is more challenging and the lack of consensus on acceptable nomenclature is due to confusion generated by obscurities regarding their origins. For instance, the presence of the plasma membrane shed as MVs is not clear in plants. On the other hand, the presence of exosome-like particles in plants has been reported. The fusion of MVBs with the plasma membrane and subsequent release of the small vesicles in the extracellular fluid, apoplast, was first reported in 1967 in a carrot cell culture [7], which is earlier than the observation of exosomes in rat reticulocytes [8]. Since then, plant EVs have been isolated from apoplastic fluid and observed by transmission electron microscopy [9]. In 2017, the evidence of critical roles of plant EVs separated from apoplast in plant defense system was corroborated [10][11], raising the interest to investigate intercellular communication via EVs in plants. Despite accumulating observations of exosomes in plants, it remains unanswered how the nanoparticles can overcome the barrier of the cell wall [12]. Aside from unclear aspects of the biology of plant exosomes, what makes the characterization of the particle more confusing is that only a small portion of studies on PDNVs have appropriately purified particles from apoplastic fluids and thus, few are indeed EVs. Most of the studies have collected particles from fruit/leaf/root juice made by gentle pressing or harsh grinding which would recover not only EVs, but also artificial membranous vesicles as well as nanovesicles which may not be of extracellular origin, such as microsomal fraction [13]. Indeed, Liu et al. directly compared the EVs isolated from apoplastic space with nanovesicles isolated from blending of the model plant *Arabidopsis thaliana* and showed some distinctions between the two types of particles. Although both were similar in size uniformity, membrane charge, and shared some well-known EV proteins, such as annexins, soluble N-ethylmaleimide-sensitive factor attachment protein receptors, and glycosylphosphatidylinositol-anchored proteins, EVs had narrower size range with different density distribution [14]. Furthermore, EVs were more readily taken up by OVCAR5 cancer cells than the leaf-derived nanoparticles, suggesting different fusion efficiency [14]. Despite the

blatant error, when considering complicated processes of isolating EVs from apoplast and about 700-fold lower yield than nanovesicles isolated from disruptive blending [14], it is anticipated that future studies will adhere to the general nanovesicle isolation protocol of not distinguishing cellular origin. Thus, the nomenclature should be taken with caution and the term “PDNVs” is used instead of “PDEVs”.

3. Isolation and Characterization Methods of PDNVs

The plant sample collection method varies by studies. Most studies simply indicated that the edible plants they used were purchased from a local market and identified the region. Others described details and took environmental factors into account. For example, Liu et al. described the origin of seeds they obtained and grew their garlic chive in a greenhouse with controlled temperature and light cycle [15]. Furthermore, to keep the maturation levels of leaves constant, they harvested leaves bi-weekly [15]. Perut et al. picked their strawberry samples from their university experimental farm [16]. To ensure the maturation level was comparable across samples, they harvested fully matured strawberries and stored them at −80 °C until analysis [16]. To avoid any confusion and to ensure reproducibility between studies, future work may need to clearly describe farming and harvesting conditions such as climate, region, and degree of maturation. At the same time, it would be interesting to investigate how such conditions alter the quantity and the quality of PDNV production. For instance, Logozzi et al. compared PDNVs grown on organic farms with those in conventional farms and found the former results in greater yield and total anti-oxidant capacity [17].

Pre-processing steps for PDNVs are largely the same with a slight modification depending on the type of fruits or the structure of vegetables. For example, fleshy fruits, such as apple [18][19], blueberry [20][21], orange [22][23], lemon [24], and grapefruit [25][26], having high water content, were crushed/smashed and then homogenized using a blender or squeezed manually or pressed using a juicer. The collected juice was then processed to isolate nanoparticles. Dry fruit, such as nuts, was homogenized with a blender and then mixed with PBS before centrifugation [27]. Similarly, corn was homogenized with distilled water [28]. Oat bran meal was dissolved in PBS and then incubated in a 37 °C water bath for 30 min for supernatant collection and subsequent centrifugation [29]. Root vegetables, such as carrot [30], garlic [31][32][33], ginger [34][35][36][37][38][39][40][41], ginseng [42][43], and turmeric [44], and leafy vegetables including cabbage [45], were prepared similarly to fruits by blending them with or without additional PBS pre- or post-grinding.

Isolation methods of PDNVs follow those established for mammalian EVs as they are considered to be universal tools. Ultracentrifugation (U/C) followed by purification using sucrose-gradient centrifugation is the most common extraction method. Particle size-based isolation methods, such as size-exclusion chromatography (SEC), ultrafiltration (U/F), and tangential flow filtration, are standards as well. Precipitation by polyethylene glycol or commercial isolation kits (e.g., ExoQuick™) is available for extraction. Immunoaffinity is another standard method for capturing mammalian exosomes, but due to high cost, potential exclusion of other subpopulations of nanovesicles, and absence of established surface protein markers and their antibodies for PDNVs, it is not preferred for PDNV isolation [46].

4. PDNVs as a Nanocarrier

For administrated bioactive molecules to exert their biological effects as intended, they must endure an unfavorable physiological environment and reach their target site in a proper amount at the right time. A wide range of drug delivery system techniques has been devised to enhance the efficacy of therapeutic agents, including nanocarriers. Organic, inorganic, metallic, and polymeric nanostructures such as liposomes, solid lipid nanoparticles, dendrimers, and micelles are examples of nanomaterials on the market or under pre-clinical investigation [47]. Nanocarriers offer several advantages over conventional forms of drugs in that cargoes are protected from unwanted degradation and show improved retention and penetration to tissue. However, application to clinical settings is impeded by several issues such as cytotoxicity [48], ecotoxicity [49][50], and mass production at affordable prices. For these reasons, naturally occurring EVs emerged as an alternative to artificially synthesized vehicles. EVs can cross natural barriers such as the blood-brain barrier [51], which is an ideal trait for carrier molecules. Second, EVs can circulate in the system for a relatively long term as they are encapsulated and protected from enzymatic degradation by the lipid bilayer structure [52]. Thus, cargoes stay stable in circulation. Lastly, they can be immunological because they are from biocompatible cells, making them less likely to trigger immune responses [53]. When compared with mammalian cell-derived EVs, PDNVs could offer more advantages because many mammalian, cell-derived EVs are engaged in tumor biology, and their potential biohazards are not clearly identified yet. In addition, mammalian cell cultures require animal components, including fetal bovine serum, which can cause critical safety problems in clinical applications [54]. Furthermore, PDNVs are more cost-effective than mammalian, cell-derived EVs. For instance, the average yield of cabbage-, red cabbage-, and carrot-derived nanovesicles were 1.504×10^{11} particles/g, 1.098×10^{11} particles/g, and 3.24×10^{11} particles/g each [30][45], while the retail prices of cabbage, red cabbage, and carrot reported by the United States Department of Agriculture in 2016 are only \$0.001367/g, \$0.002249/g [45], and \$0.001698/g, respectively. Vesicles could be obtained as a by-product of crops, such as roots or leaves, which are otherwise non-profitable, providing profits. For instance, nanovesicles obtained from tomato root exudates without infections harbored similar proteins typically present in plant apoplastic vesicles and exerted anti-fungal activity in vitro [55]. In terms of stability, grapefruit-derived nanovesicles were resistant to in vitro digestion by gastric pepsin and pancreatic and bile extract solution [56]. Ginger-derived nanovesicles were also tested for stability by incubating them in a stomach-like solution (pepsin solution in pH 2.0) or first in the stomach-like solution and then in small intestine-like solution (bile extract and pancreatin solution with pH adjusted to 6.5) [34]. The results showed the ginger-derived nanovesicles were stable in those solutions with a slight reduction in size and zeta potential changed according to the surrounding pH: negative charge in the PBS and intestine-like solution; and slightly positively charged in the stomach-like solution [34]. Turmeric-derived nanovesicles also maintained nano-scale size under different pH solutions with their size increased, undergoing similar zeta potential changes as ginger-derived nanovesicles [44]. These collectively suggest PDNVs would survive a harsh environment in the GI tract when orally consumed in food form or purified form due to their membrane versatility.

5. Modification of PDNVs to Better Serve as a Nanocarrier

PDNVs can undergo additional processes to incorporate therapeutic cargoes into the interior. The loading methods include co-incubation, electroporation, sonication, chemical transfection, freeze-thaw method, and extrusion [57][58]. Co-incubation is helpful for the cargoes that can diffused into the interior of the vesicles through the membrane and is relatively simple, but its loading efficiency is low. Incubation of cabbage-derived nanovesicles with miRNA and transfection reagent or incubation of cherry-derived nanovesicles with miRNA on ice successfully incorporated miRNA into the interior [45][59].

The surface of PDNVs could also be modified to achieve targeted delivery, increased stability, and efficient uptake. One example of nanoparticle surface modification is polyethylene glycol coating (PEGylation), which decreases immunogenicity and increases systemic circulation time [60]. When nanovesicles from *Asparagus cochinchinensis* were PEGylated, vesicles were retained in circulation for a prolonged time and accumulated more in tumor tissue without side effects. Patching of heparin-cRGD (tripeptide Arg-Gly-Asp motif) peptide conjugates onto lemon-derived nanovesicles [61] or patching of doxorubicin loaded heparin-cRGD- based nanoparticles onto the surface of grapefruit-derived nanovesicles [62] are another examples of surface modification. Since RGD motifs are recognized by αvβ3 integrins in proliferating endothelium of tumors [63], such surface modification could improve the tumor-targeting capacity of PDNVs. Moreover, heparin helps to increase the stability and in vivo retention time due to its anti-complement activation capacity [64].

Chen et al. [65] used tris (2-carboxyethyl) phosphine (TCEP) to selectively reduce disulfides on proteins of grape and ginger-derived nanovesicles. TCEP is a mild reducing agent that does not react to other molecules, such as phospholipids, and thereby, membrane integrity is preserved. The TCEP-reduced nanovesicles were then reacted with maleimide derivatized transferrin, a cancer-targeting ligand, increasing the target-specificity of EVs [65].

6. Plant-Derived Lipid Reassembled Particles

In order to increase the uniformity and reproducibility of nanoparticles, some studies used lipid extracts of PDNVs and reconstructed new lipid nanocarriers. Caveats of this recombinant method are increased complexity of nanocarrier preparation procedures and potential loss of inherently contained cargoes, and thus losing key properties of unmodified PDNVs. Despite these disadvantages, Wang et al. showed that reassembled grapefruit-derived nanoparticles (GNPs) could be used to deliver chemotherapeutic agents (JSI-124, paclitaxel) and siRNAs to cancer cells, such as brain tumor cells (GL26) and colon cancer cells (CT26, SW620) [66]. In other studies, GNPs were further modified by the leukocyte plasma membrane coating or folic acid coating to further confer a desirable multilayer surface [67][68]. Indeed, folate coating enhanced targeted delivery to folate receptor-positive brain tumor tissue and leukocyte plasma membrane coating augmented delivery specificity to inflammatory tissue [67][68].

A ginger-derived nanovector loaded with RNAs was produced by ginger-derived nanovesicle lipid extraction and suspension with RNAs, followed by UV irradiation and sonication [69]. TEM analysis showed reconstituted nanovectors and parental nanovesicles were similar morphologically and FACS analysis showed the nanovector is taken up by both h F4/80+ macrophages and EpCAM+ lung epithelial cells after intratracheal injection [69], showing

that the reconstituted nanovector retains its ability to be efficiently taken up. In terms of transfection efficiency, the nanovector loaded with aly-miR396a-5p delivers the miRNA more efficiently than the parental, ginger-derived nanovesicle or polyethylenimine and less than RNAiMAX in A549 cells. The superior delivery efficiency of the miRNA compared with gold nanoparticles after intratracheal injection again supports the promising application of the ginger-derived nanovector [69]. Furthermore, when the effect of lipids was tested by manipulating the level of predominant lipids PA, PC, or PE in the ginger-derived nanovector, additional PE in the nanovector resulted in increased uptake by A549 cells, whereas PA and PC inhibited uptake [69]. Other studies that used a ginger lipid-derived nanovector also confirmed the superior ability of particles to liposome in terms of biocompatibility and efficient delivery of cargoes such as siRNA and chemotherapeutic agent doxorubicin [70][71].

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