REVIEW

Plant derived edible nanoparticles as a new therapeutic approach against diseases

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ABSTRACT

In plant cells, nanoparticles containing miRNA, bioactive lipids and proteins serve as extracellular messengers to mediate cell-cell communication in a manner similar to the exosomes secreted by mammalian cells. Notably, such nanoparticles are edible. Moreover, given the proper origin and cargo, plant derived edible nanoparticles could function in interspecies communication and may serve as natural therapeutics against a variety of diseases. In addition, nanoparticles made of plant-derived lipids may be used to efficiently deliver specific drugs. Plant derived edible nanoparticles could be more easily scaled up for mass production, compared to synthetic nanoparticles. In this review, we discuss recent significant developments pertaining to plant derived edible nanoparticles and provide insight into the use of plants as a bio-renewable, sustainable, diversified platform for the production of therapeutic nanoparticles.

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Introduction

When seeking to develop new therapeutic approaches against diseases, researchers must overcome numerous challenges, including issues with delivery, potential off-target effects, safety, toxicity, large-scale production costs, and tissue specificity. Our group and others have recently demonstrated that artificially synthesized nanoparticles may be used to target low doses of drugs (e.g., siRNAs, proteins and peptides) to specific cell types and tissues.¹⁻¹⁴ However, the nanoparticles synthesized to date have two major limitations: i) each constituent of the synthesized nanoparticles must be examined for potential *in vivo* toxicity before clinical application; and ii) the production scale is limited. The use of nanoparticles derived from natural sources may overcome these limitations of synthetic nanoparticles.

Exosomes are endogenous nanoparticles produced by mammalian cells

Among the diverse vesicles released by animal cells, exosomes have recently received attention as having potential for diagnostic and therapeutic applications.¹⁵⁻²² Exosomes are small (30–100 nm)

membranous nanovesicles of the endocytic cellular pathway. Exosomes can be formed and released into the extracellular environment through three stages: i) formation of endocytic vesicles by invagination of the plasma membrane; ii) inward budding of the late endosomal membrane, which gives rise to multivesicular bodies (MVBs); and iii) fusion of MVBs with the plasma membrane and release of the vesicular contents (exosomes).²³ A variety of mammalian cells, including immune cells,²⁴ epithelial cells,²⁵ platelets,²⁶ dendritic cells,²⁷ mast cells²⁸ and cancer cells,²⁹ have the capacity to release exosomes. In addition, exosomes also have been found in physiological fluids, such as serum,³⁰⁻³² urine,³³⁻³⁵ breast milk,^{36,37} saliva³⁸⁻⁴⁰ and malignant effusions.^{41,42}

Exosomes comprise a lipid bilayer membrane plus membrane-associated proteins, and contain cytosolic components such as mRNAs, miRNAs and proteins. Exosomes may putatively attach to or fuse with the membrane of a target cell, and thereby deliver exosomal surface proteins and cytosolic components to the cell.⁴³ Finally, exosomes might also be internalized by a recipient cell through a mechanism such as endocytosis, enabling them to serve as extracellular

messengers.⁴⁴ It has been demonstrated that exosomes participate in cell-cell communication by transferring their bioactive contents to distant cells, and they have been suggested to regulate gene and protein expression levels in recipient cells. Exosomes have been well characterized, and may be identified using exosomal protein markers such as CD63, CD9, CD81 and Hsp70.⁴⁵ They can be isolated from conditioned cell culture media or bodily fluids by differential centrifugation, filtration plus centrifugation, immuno-affinity or size exclusion chromatography, polymer-based precipitation, and microfluidic technologies that utilize this principle.⁴⁶⁻⁵⁰ Each method has its advantages and disadvantages, requires different sample pre-processing steps, and produces exosomes preparations of varying purity and quality.

Plants as nanofactories

Although exosomes have not previously been documented in plants, vesicle-mediated plant-cell communication has been studied.^{51,52} Recently, it has been

demonstrated that nanoparticles from the juice of grapefruits, grapes, ginger, carrots and tomatoes may be isolated by using an eco-friendly protocol⁵³ (Fig. 1). Different characterization methods have shown that these nanosized vesicles have structures similar to those of mammalian-derived exosomes. Plant-derived edible nanoparticles (PDENs) may be involved in plant cell-cell communication, could potentially regulate the innate immunity of plants,^{54,55} and may also transport mRNAs, miRNAs, bioactive lipids, and proteins into animal cells. These observations suggest that the use of plants as nanofactories for the fabrication of medical nanoparticles could represent a new approach in nanomedicine.

Isolation of plant derived edible nanoparticles

For the isolation of plant derived edible nanoparticles, in contrast to other methods available, differential ultracentrifugation plus density gradient centrifugation is the “gold standard.” Plants are ground to juice in a mixer; low-speed centrifugation (e.g., $1,000 \times g$)

Figure 1. Representative plants derived edible nanoparticles and drug loaded plant derived edible lipid nanoparticles with specific target properties from ginger (A), grapefruit (B), carrot (C), grape (D). Edible plants were purchased from local market; plants derived edible nanoparticles can be isolated from edible plants using differential ultracentrifugation plus density gradient centrifugation. For plants derived edible lipid nanoparticles fabrication, lipids were first isolated from plants derived edible nanoparticles, and then a standard method based on hydration of lipid film similar with liposome fabrication was employed. Note: lipid nanoparticles derived from carrot and grape have not been reported.

is used to remove large particles and plant fibers; medium-speed centrifugation (e.g., $10,000 \times g$) is used to remove large debris and intact organelles; and high-speed centrifugation (e.g., $150,000 \times g$) is used to pellet the exosome-like nanoparticles. The quantity and quality of the obtained nanoparticles are sensitive to the g force,^{22,23,35} rotor type, and angle of rotor sedimentation. Moreover, this method also sediments other vesicles, proteins and/or protein/RNA aggregates. Thus, a subsequent sucrose density gradient step is used to separate the plant derived edible nanoparticles from contaminants of different densities. Gradient ultracentrifugation requires an extended centrifugation time (1–5 h), but provides a more purified edible plant nanoparticle isolate than ultracentrifugation alone.

Biochemical characterization of plant derived edible nanoparticles

The RNA, lipid and protein contents in plant derived edible nanoparticles differ from those found in mammalian exosomes. For example, Ju et al. reported that grape exosome-like nanoparticles (GELNs) contained 96 miRNAs and 28 identified proteins.⁵⁶ In comparison, mammalian exosomes typically contain 100–300 miRNAs and more than 1,000 proteins. In terms of the lipid profile, mammalian exosomes are generally rich in cholesterol and sphingomyelin but have only low levels of phosphatidylethanolamine and the mitogenic compound, phosphatidic acid (PA). In contrast, GELNs were found to comprise 98% phospholipids (50% of which was PA) and 2% typical plant lipids (e.g., galactolipids, such as digalactosyldiacyl-glycerol and monogalactosyldiacyl-glycerol). PA has been shown to interact with mammalian target of rapamycin (mTOR), and is known to trigger cell growth and proliferation.⁵⁷ In addition, PA, mitogenic phospholipid, is highly fusogenic in the presence of calcium and has thus been postulated to induce inter-vesicular fusion.⁵⁸ Wang et al. (2014) isolated grapefruit-derived exosome-like nanoparticles (GDNs) and found that they contained 137 proteins and had a lipid profile that differed from that of GELNs. Mass spectrometry-based protein profiling of GDNs revealed that a number of the proteins were responsible for regulating carbohydrate/lipid metabolism. The lipidomic

data indicated that GDNs were enriched for phosphatidylethanolamine (45.52%) and phosphatidylcholine (28.53%), with particularly high levels of phosphatidylethanolamine (34:2) and phosphatidylcholine (34:2). These differences of plant-derived exosome-like nanoparticles may play important roles in interspecies communication, such as long-distance communication throughout the entire mammalian digestive tract.

Ultrastructure of plant derived edible nanoparticles

Transmission electron microscopy (TEM) has been used to characterize plant derived edible nanoparticles. Although this technique does not allow researchers to obtain additional biochemical and mechanical information, it is the major method used for ultrastructural analysis at the subcellular level.^{59–61} For TEM analysis, exosome-like nanoparticles can be resuspended in a water-soluble solution (e.g., PBS, HEPES, Tris-HCl, and deionized water), spotted onto a carbon-coated grid, fixed with 1% glutaraldehyde, stained with 2% phosphotungstic acid, and visualized. Our group has found that high-resolution photos can be obtained using plant derived edible nanoparticles suspended in deionized water. Although TEM-based imaging of plant nanoparticles may be compromised by the loss of membranes during the dehydration and centrifugation/embedding steps, the ultrastructures of various plant derived edible nanoparticles (e.g., from grapefruit, carrots, ginger, and tomatoes) have been reported.⁶² Atomic force microscopy (AFM) can also be used to study the sizes and structures of individual plant derived edible nanoparticles. AFM is a very high-resolution type of scanning probe microscopy (SPM) that has a demonstrated resolution on the order of fractions of a nanometer, which is more than 1,000 times better than the diffraction limit of optical microscopy.^{63–65} For preparation of the samples, purified plant derived edible nanoparticles are diluted in deionized water, adsorbed to freshly cleaved mica sheets, rinsed two or three times with deionized water, and dried under a gentle stream of nitrogen. Our group has used AFM to characterize plant derived edible nanoparticles obtained from ginger. The observed AFM phase contrast of ginger derived nanoparticles indicate substructure details and non-homogenous surface, which is tentatively attributable to the presence of proteins and/or mRNA enclosed inside the

highly dense lipid membrane, consistent with previous proteomic and RNA analysis of saliva and other exosomes population from animals.⁶⁶

Determining the charges and sizes of plant derived edible nanoparticles in solution

Dynamic light scattering (DLS, also known as photon correlation spectroscopy or quasi-elastic light scattering) can be used to determine the size-distribution profiles of small particles in suspension. In DLS, fluctuations of the scattered light intensity (which are due to the Brownian movement of the particles) are correlated to get information about particle size. Since DLS is noninvasive and highly sensitive, and requires only very small sample volumes, it has been widely adopted in many scientific fields. It is currently the gold standard for quickly and accurately determining the size distribution of suspensions.⁶⁷⁻⁷⁰ The sizes and zeta potentials of plant derived edible nanoparticles can be determined by DLS.⁶² The median diameter of a nanoparticle is considered to be a key parameter for the therapeutic efficacy of a nanoparticle. Plant derived edible nanoparticles obtained from grapefruit, ginger, tomatoes and garlic reportedly have plant-specific monodispersed size distributions, with median diameters of ~400 nm for edible grape nanoparticles, and ~250 nm for edible grapefruit and ginger nanoparticles. In contrast, edible carrot nanoparticles have two such distributions, one ~100 nm and the other ~1,000 nm. Plant derived edible nanoparticles also show negative zeta potentials (−49.2 to 1.52 mV) of relatively low value at pH ~6, indicating that they exhibit mutual repulsion and no tendency toward aggregation.⁷¹

Plant derived edible nanoparticles with therapeutic effects

Plant derived nanoparticles appear to play a role in interspecies communication, and have been postulated to function against inflammatory diseases and cancers.^{56,62} Recently, it has been shown that oral administration of grape exosome-like nanoparticles to mice reportedly triggered proliferation of cells in the intestinal epithelium, and was associated with intestinal stem cell proliferation throughout the intestine and colon.⁵⁶ Furthermore, orally administered grape exosome-like nanoparticles appeared to be resistant to degradation by saliva, the acidic environment of the stomach, and

the highly active proteolytic enzymes present along the intestinal tract. These recent observations suggested that edible-plant-based nanoparticles can be orally delivered to the intestine, where they may be taken up by intestinal cells and exert functions such as intestinal regeneration. Plant derived edible nanoparticles that naturally target colonic tissues and have anti-inflammatory properties may therefore represent a novel natural and nontoxic delivery system that could easily be scaled up for the treatment of digestive tract diseases such as intestinal bowel disease (IBD).

Ju et al.⁵⁶ provided the first report of interspecies communication between plant and gut host cells by demonstrating that GELNs have unique transport properties and biological functions. They showed that GELNs can penetrate the intestinal mucus barrier, be taken up by mouse intestinal stem cells, and significantly induce the activation of Lgr5⁺ intestinal stem cells through the Wnt/ β -catenin pathway. They also showed that oral administration of GELNs had protective effect on dextran sulfate sodium (DSS)-induced colitis mice by triggering the growth and proliferation of intestinal stem cells which have a crucial role in regulating intestinal epithelial cells differentiation and are required for intestinal tissue homeostasis and repair.

Wang et al.⁷² illustrated that grapefruit-derived nanovesicles (GDNs) were selectively taken up by intestinal macrophages and ameliorated DSS-induced mouse colitis. The anti-inflammatory effects of GDNs were mediated by up-regulating the expression of heme oxygenase-1 (HO-1) and inhibiting the productions of IL-1 β and TNF- α in intestinal macrophages. The authors also confirmed that GDNs are biocompatible, biodegradable, and stable across a wide range of pH values, and proposed that they could be developed as a novel oral drug delivery system. Based on this, the authors incorporated an anti-inflammatory drug (methotrexate, MTX) into GDNs,⁵⁶ and found that the encapsulated drug showed a lower toxicity than free MTX and exhibited remarkably higher therapeutic effects against DSS-induced colitis in mice. Their finding demonstrated that GDNs can serve as immune modulators in the intestine and promote homeostasis of intestinal macrophages. These nanoparticles thus show a potential for development as an oral delivery system for small molecule drugs aimed at attenuating inflammatory responses in human disease.

Mu et al.⁶² isolated and characterized plant derived edible nanoparticles from ginger, carrots, grape and

grapefruit, and found that they were similar to mammalian-derived exosomes in terms of their sizes and structures. They contained proteins, lipids, and miRNAs, and were found to be taken up by intestinal macrophages and stem cells. The results obtained from PDEN-transfected macrophages indicated that ginger PDENs preferentially induced the expression levels of the antioxidant, heme oxygenase-1 (HO-1), and the anti-inflammatory cytokine, IL-10. Nuclear factor- κ B (NF- κ B) as a key regulator of *HO-1* gene, has an important role in anti-inflammation and anti-oxidation, which can be used as an indicator for regulating the cellular anti-inflammatory response. The authors also confirmed grapefruit, ginger, and carrot PDENs could activate nuclear translocation of NF- κ B.⁶² Furthermore, analysis of the intestines of β -catenin/TCF/LEF reporter transgenic mice treated with the various PDENs revealed increased numbers of activated β -catenin in intestinal crypts. This suggests that PDENs have dominated effects on modulating Wnt/TCF/LEF activity and play an important role in gut homeostasis.^{73,74} Thus, PDENs-mediated interspecies communication may involve the activations of anti-inflammatory cytokines, antioxidants, and Wnt signaling, which are crucial for maintaining intestinal homeostasis.

In addition to the apparent therapeutic activity of PDENs against inflammatory diseases, plant derived exosome-like nanoparticles may also affect cancer progression. Raimondo et al.⁷⁵ isolated vesicles from lemon juice (*Citrus limon* L.) by differential ultracentrifugation; this fraction had a density of 1.15 to 1.19 g/ml and was characterized by a specific proteomic profile. The authors showed that these nanoparticles inhibited cancer cell proliferation in different tumor cell lines *in vitro* by activating TRAIL-mediated apoptotic cell death. Furthermore, they demonstrated that lemon nanovesicles suppressed CML tumor growth *in vivo* by specifically targeting the tumor site and activating TRAIL-mediated apoptosis. This study suggested for the first time that plant-edible nanovesicles could be used to treat cancer.

Plant derived edible lipid nanoparticles as novel nanoparticle drug carriers

Lipids are currently the preferred carriers for drug delivery, but synthetic liposomes and lipid nanoparticles can yield undesirable effects, such as cell stress,

inflammasome activation, and apoptosis.⁷⁶ The use of nontoxic “natural” nanovectors assembled from plant nanoparticle-derived lipids has been suggested as a feasible alternate strategy for *in vivo* drug delivery (Fig. 1).⁷⁷ Experiments in our group using ginger nanoparticles (diameter, 180 nm, zeta potential, -5 mV) demonstrated the feasibility of generating “natural” nanovectors from ginger nanoparticles-derived lipids. The generated lipid nanoparticles had no cytotoxicity or negative effect on intestinal barrier function, suggesting that they may be useful for efficient *in vivo* drug delivery.⁷⁷

Notably, PDEN-derived lipid nanoparticles are believed to be excellent candidates for delivering various therapeutic agents. Wang et al.⁷⁷ showed that nanoparticles made of grapefruit-derived lipids, which they called grapefruit-derived nanovectors, could deliver chemotherapeutic agents, siRNAs, DNA expression vectors, and proteins to different cell types. They also used grapefruit-derived nanovectors to co-deliver therapeutic agents plus folic acid, and reported that this strategy significantly increased the targeting efficiency to cells that also express folate receptors. These nanovectors were further demonstrated to enhance the chemotherapy-induced inhibition of tumor growth in CT26- and SW620-cell-derived tumors in mice. The authors showed that grapefruit-derived nanovectors were less toxic than nanoparticles made of synthetic lipids and did not cross the placental barrier when injected intravenously into pregnant mice, suggesting that they could prove useful for drug delivery.

In another excellent study, Wang et al.⁷⁸ coated their grapefruit-derived lipid nanoparticles (GDNVs) with inflammatory-related receptor enriched membranes of activated leukocytes (IGNVs). Using different mouse models of inflammation-driven disease, they demonstrated that IGNVs showed better targeting of inflamed tumor tissues compared with GDNVs. Moreover, this homing to inflamed tissues was significantly inhibited by the blockade of LFA-1 or CXCR1/CXCR2 on the IGNV membranes.

Summary

Edible plant-derived nanoparticles may possess intrinsic therapeutic activities against specific diseases. Researchers are currently seeking to select and characterize specific populations of plant derived edible

nanoparticles that have such activities and/or examine their intrinsic targeting. It is anticipated that such investigations will significantly contribute to the development of natural nano-medicines. Plant-derived lipid nanoparticles may act as natural drug carriers that can efficiently target drug delivery to a specific location of the human body. Thus, plant derived edible nanoparticles and plant-derived lipid nanoparticles, which offer multiple benefits (e.g., nanoparticle size, low toxicity, good tissue-specific targeting, excellent environmental safety, and a significant potential for large-scale production) could serve as next-generation therapeutic delivery systems for the treatment of inflammatory diseases, cancers, and other diseases.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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