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Characterization of the MicroRNA Profile of Ginger Exosome-like Nanoparticles and Their Anti-Inflammatory Effects in Intestinal Caco-2 Cells

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ABSTRACT: Plant-derived exosome-like nanoparticles (PELNs) have been shown to enter mammalian cells for disease treatment. Although abundant miRNAs are contained in ginger exosome-like nanoparticles (GELNs), little is known about their type and function. Herein, we extracted GELNs with desirable particle sizes (156 ± 36 nm) and a negative surface charge (−26.6 ± 5 mV). The miRNA profiles in ginger and GELNs were analyzed using high-throughput sequencing, and the results of the sequencing were validated by real-time quantitative polymerase chain reaction (RT-qPCR). There were 27 miRNAs with higher expression levels in the GELNs, and they were mainly involved in the regulation of inflammatory and cancer-related pathways. Furthermore, GELNs could be specifically internalized by intestine cells via caveolin-mediated endocytosis and micropinocytosis, as well as counteract lipopolysaccharide (LPS)-induced inflammation by downregulating NF-$\kappa\beta$, IL-6, IL-8, and TNF-α expression. Importantly, the positive effects were further proved to be possibly related to the miRNAs enriched in the GELNs. Overall, these results indicated that PELNs could target human digestive organs and play a cross-kingdom physiological regulation role through miRNAs.

KEYWORDS: *ginger exosome-like nanoparticles, miRNA profiles, small RNA sequence, mechanism absorption, intestinal inflammatory*

INTRODUCTION

On a worldwide scale, ginger (the rhizome of *Zingiber officinale*), a perennial plant, is widely used as a dietary condiment in food and beverages.[1] Studies have reported that the beneficial effects of ginger include decreasing risk of platelet aggregation, reducing risk of endothelial dysfunction, and managing digestive disorders.[2] Ginger is not only rich in dietary fiber, volatile compounds, and nonvolatile biologically active substances such as gingerols, shogaols, and zingerone but also tiny vesicles that are similar to exosomes.[3,4]

Exosomes are cell-derived extracellular vesicles of endosomal origin that are naturally released by most cells and are fundamental for intercellular communication.[5] Plant-derived exosome-like nanoparticles (PELNs) are 30−300 nm extracellular nanovesicle-like mammalian exosomes, have the lipid-bilayer membrane structure, and encapsulate proteins, lipids, DNA, and various RNA species.[6] Recently, increasing evidence demonstrated that PELNs play critical roles in multiple pathophysiological processes such as maintaining intestinal homeostasis and affecting intestinal function.[7−9] For example, Chen et al. reported that exosome-like nanoparticles from ginger rhizomes could strongly inhibit NLRP3 inflammasome activation in primary macrophages by blocking the assembly steps.[10] Zhang et al. found that the nanoparticles derived from ginger can target the inflamed intestinal mucosa to improve the prevention and treatment of inflammatory bowel disease.[11] These observations indicated that PELNs might be valuable bioactive factors for health. However, the mechanisms that underlie these health benefit effects remain largely unknown.

When we eat various plant foods, PELNs are naturally absorbed by special host cells and proceed with the transmission of information.[12] The human gastrointestinal tract inevitably comes into contact with PELNs, so the interaction of PELNs−intestine cells has aroused wide attention. At present, progress remained slow in exosome-mediated intestinal disease therapies. This is due primarily to the limited understanding of the exosome−intestine cell interaction. Recipient cells were reported to internalize exosomes by various mechanisms such as receptor-mediated pathways, macropinocytosis, phagocytosis, and membrane fusion.[13] Various cells have been demonstrated to take up exosomes from the diversified cell with different extents.[14] However, whether the mechanism of how ginger exosome-like nanoparticles (GELNs) were absorbed by intestine cells remains unknown. Meanwhile, it is not clear whether GELNs can provide a natural means of genetic material transfer to the intestine.

The cross-kingdom functions may be achieved through the transfer of the cargo contained in PELNs, especially microRNAs (miRNAs), from donor to recipient cells and binding of exosomes to cell surface receptors.[15] miRNAs are a class of approximately 22 nucleotides of noncoding RNAs involved in regulating many biological pathways by recognizing and cleaving target mRNAs or translational repression.[16] There is

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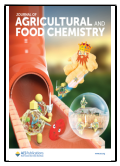

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increasing interest in the role of miRNAs in human health and diseases.[17] However, severe on-target or off-target side effects of artificial miRNAs are permanently debated.[18] Notably, miRNAs in PELNs are stable, nontoxic, and bioavailable. They can reach target tissues by transport of exosomes and show the bioactivity.[19] Research has shown that miR-18a in grapefruit-derived nanovesicles could mediate M1 macrophage along the IFN-γ/Irf2 axis to inhibit liver metastasis.[20] Another report indicated that nanovesicles derived from edible ginger were able to mediate gut microbiota and improve intestinal inflammation through miRNAs.[21] Meanwhile, high-throughput sequencing technology and bioinformatics analysis also indicated that plant-derived miRNAs have the potential as new bioactive compounds to regulate human metabolic activities and even human diseases.[22] Based on small RNA high-throughput sequencing, microRNAs derived from coconut water were identified to target the human genome and likely regulate mRNAs relevant to metabolism.[23] Hence, to better unravel the intestine health benefits of GELNs, it is important to understand microRNA profiles of GELNs and their effects on the intestinal inflammatory response.

In the present study, we first extracted GELNs from ginger using a novel method of differential centrifugation combined with an extraction kit. Then, we constructed and compared the small RNA libraries between ginger and GELNs. The highly expressed miRNAs in the GELNs, compared to ginger, were performed by target gene prediction and function analysis. In addition, using Caco-2 cells as models, we also aimed to investigate the absorption mechanism of the GELNs and their role in the inflammatory response. Our findings may provide a better understanding of ginger in terms of molecular events and physiological functions, as well as develop new application strategies for the PELNs.

METHODS

Chemicals. Ginger (*Z. officinale* Roscoe. cv. Anqiu big ginger) was purchased from RT-MART (Hefei, China). The exosome isolation kit (Cat. No: UR52121) was obtained from Umibio (Shanghai, China). The fluorescent lipophilic dyes, DIO, 4′,6-diamidino-2-phenylindole (DAPI), and PKH67, were procured from Thermo Fisher Scientific (MA). Glutaraldehyde, phosphotungstic acid, lipopolysaccharide (LPS), bicinchoninic acid (BCA) protein assay kit, RNase, amiloride, indomethacin, and chlorpromazine were acquired from Sigma-Aldrich (MO). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), nonessential amino acids, penicillin, and streptomycin were obtained from Invitrogen-Gibco (Grand Island, NY).

Isolation and Purification of GELNs. Ginger was cut into many small pieces and put into a juice extractor (Joyoung Company Limited, China). Subsequently, the juice was separated and collected. According to the manufacturer's protocol with some modulation, GELNs were isolated from ginger juice using the exosome isolation with Umibio exosome isolation kits (Umibio, Cat. No: UR52121, China). In brief, the collected fluid (30−40 mL) was centrifuged at 4000*g* for 60 min, 6000*g* for 60 min, and 8000*g* for 60 min, 12,000*g* for 60 min, and 18,000*g* for 60 min to remove pellet cells and debris, and then 20 mL of the supernatant was added to 5 mL of exosome concentration solution. Mixtures were vortexed and incubated at 4 °C for up to 2 h and then centrifuged at 18,000*g* for 60 min to precipitate nanoparticles. Nanoparticles were suspended with 1× PBS, passed through a 450 nm filter (Acrodisc), and purified further with an exosome purification filter at 16,000*g* for 10 min. Purified exosomes were kept at −80 °C until use. GELN protein content was qualified using the BCA protein assay kit with bovine serum albumin as a standard.

Characterization of GELNs. The morphological properties of the GELNs were recorded with a HT7700 transmission electron microscope (TEM) using a conventional procedure.[24] In brief, the resuspended pellets were fixed with glutaraldehyde and stained with phosphotungstic acid on carbon-coated grids. The prepared samples were observed under an electron microscope at 80 kV. A Malvern Mastersizer2000 was used to measure size and ζ-potential of GELNs as described.[25] Each sample was performed with three independent measurements.

Total RNA Preparation. In brief, the GELNs (1 mg) or ginger powder (30 mg) was mixed with the Trizol reagent (Invitrogen, CA), and then the mixture was put in the grinder and ground for 4 min. Chloroform was added to the mixture and centrifuged to promote liquid stratification. RNA in the aqueous phase was precipitated by isopropanol and ethanol. The quality and quantity of RNA were analyzed using a NanoDrop Spectrophotometer 2000c (DE) and Agilent RNA bioanalyzer chip.

Small RNA Sequencing and miRNA Identification. Sequencing libraries were prepared using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (NEB) and sequenced on an Illumina Hiseq. 2500/2000 platform. Library quality was validated on an Agilent Bioanalyzer 2100 system. The raw sequence reads were filtered by removing reads containing ploy-N, with 5′ adapter contaminants, without 3′ adapter or the insert tag, containing ploy A or T or G or C, reads smaller than 18 nt, and low-quality reads. To eliminate the noncoding RNAs (rRNA, tRNA, snRNA, etc), small RNA tags were mapped to the Rfam database, version 10.1. Filtered reads were aligned with the transcriptome of *Z. officinale* Roscoe by Bowtie. The mapped reads were used to look for known miRNAs based on the miRBase database, version 21.0. To predict the novel miRNAs, miREvo and mirdeep2 software were used.

Differential Expression of miRNAs. The expression levels of known and new miRNAs were estimated by TPM (transcript per million) according to the following criteria. The normalization formula is expressed as follows: Normalized expression = Actual miRNA read count/Total count of clean reads × 1,000,000.[26] The fold change between samples was calculated as follows: Fold change = log 2 (sample 1 read count/sample 2 read count). The *P*-value was inferred by the Bayesian method.[27] The *P*-value $\leq$ 0.05 and |fold change| $\geq$ 1 were set as the threshold of the differentially expressed miRNAs using the DEGseq R package (1.8.3).[28]

Target Prediction and Functional Annotation of miRNAs. Miranda software was used to predict targets of partial differentially expressed miRNAs in the human genome with the default parameters.[29] Functional analysis of target genes was mainly based on the GO database and KEGG database.

Real-Time Quantitative Polymerase Chain Reaction (PCR). The same amount of RNA of each sample, including 10 pmol of synthetic Caenorhabditis Elegans miR (cel-miR-39) for each sample, was reverse-transcribed using the miRNA first-strand cDNA synthesis (B532451, Sangon Biotech, China). cDNA samples were preamplified by the microRNAs qPCR kit (B532461, Sangon Biotech, China). Relative miRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.[30] The qPCR experiments were conducted with two pooled biological replicates and three technical replicates. The primer's information is shown in Table S5 (Supporting Information).

Labeling of GELNs. The PKH26 fluorescent cell linker kit was used for fluorescent labeling of GELNs. The same amount of samples was mixed with the dye and incubated at 37 °C for 30 min. Then, 1× PBS was added to the mixture and centrifuged for 2 h at 100,000*g* to remove the uncombined free dye. Labeled GELNs were resuspended by 1× PBS for further experiments.

Endocytosis Experiments. The human epithelial colorectal adenocarcinoma Caco-2 cell line was obtained from Procell Life Science and Technology company (Wuhan, China). Caco-2 cells were grown in DMEM supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% penicillin, and 1% streptomycin in 5% CO_2 atmosphere at 37 °C. Caco-2 cells were seeded on confocal dishes (15 mm) and incubated overnight. Then, the fresh growth medium containing labeled GELNs (2 μg/mL) was cultured with cells in an incubator. After 0.25 h or 6 h of incubation, Caco-2 cells were fixed with 10% formaldehyde and stained with DIO and DAPI.

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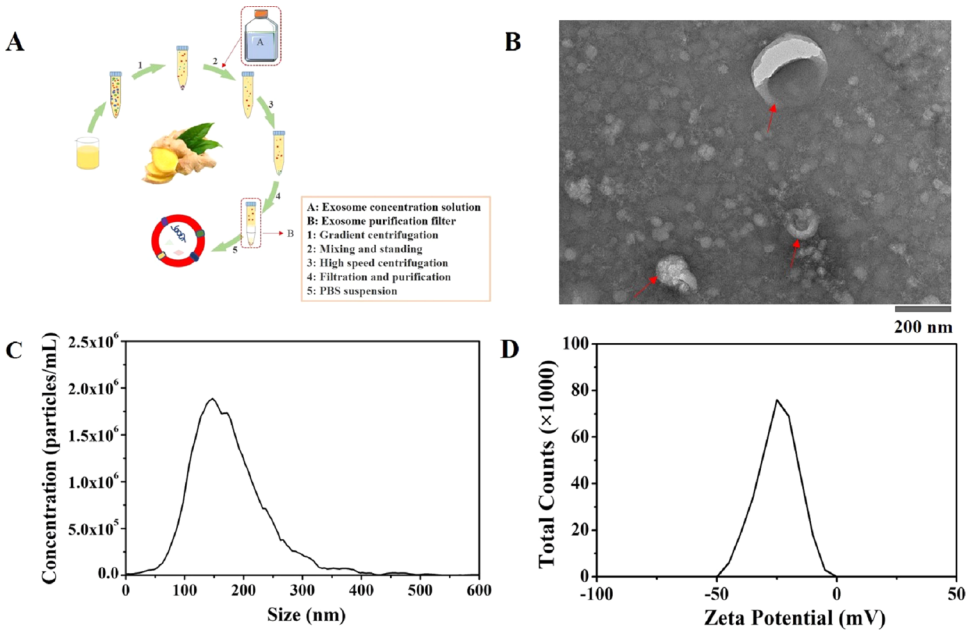

Figure 1. (A) Schematic diagram of extraction of the GELNs from ginger. (B) Morphology of the GELNs was analyzed by TEM. Scale bar represents 200 nm. The red arrows are the GELNs. (C, D) Particle size and surface charge of the GELNs.

Table 1. Sequencing Results for Small RNAs from Two Libraries (Ginger and GELNs)[a]

sample	total reads	N % > 10%	low quality	5 adapter contamine	3 adapter null or insert null	with ploy A/T/G/C	clean reads
ginger	23 754 402 (100%)	1398 (0.01%)	83 657 (0.35%)	41 650 (0.18%)	1 297 431 (5.3%)	72 892 (0.31%)	22 257 372 (93.85%)
GELNs	15 344 646 (100%)	167 (0.00%)	19 428 (0.13%)	16 550 (0.1%)	469 517 (3.06%)	2707 (0.02%)	14 836 275 (96.69%)

[a]Data are expressed as mean ± SD, $n = 3$.

Confocal microscopy (Olympus FV1, 000, Japan) was used to capture the images. To explore the mechanisms associated with GELN absorption, endocytosis inhibitors[8] (amiloride 50 μmol/L, indomethacin 100 μmol/L, chlorpromazine 12.5 μmol/L) were preincubated or co-incubated with Caco-2 cells for 1 h, and then, labeled GELNs were added for another 6 h of incubation at 37 °C.

In Vitro Anti-Inflammatory Activity of GELNs. As reported previously, lipopolysaccharide (LPS) was used to induce cellular inflammation.[31] Caco-2 cells were seeded onto 24-well plates at a density of 1×10^5 cell/well and were incubated with LPS (5 μg/mL), LPS + GELNs (60 μg/mL), and LPS + D-GELNs (60 μg/mL) for 24 h. D-GELNs were the RNA-depleted GELNs. The method of RNA depletion in GELNs referred to the previous research.[10] Total RNA of Caco-2 cells was extracted using the Trizol isolation reagent. RNA was reverse-transcribed and determined by a primer-script RT-PCR kit and real-time PCR using an SYBR Premix Ex Taq according to the manufacturer's instructions. The real-time PCR was performed as per the following program: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 1 min. Relative quantifications were normalized against GAPDH and β-2-microglobulin.[32] The primer's information is presented in Table S6 (Supporting Information).

Statistical Analysis. The experimental results were analyzed using Excel 2019 and were presented as mean ± standard deviation (SD). Statistical analysis was performed with Origin Pro 2019 software. All data were analyzed using one-way analysis of variance (ANOVA) with a Tukey's post hoc test, and statistical differences were considered significant at * $P < 0.05$ or ** $P < 0.01$.

RESULTS

Analysis of Exosome-like Nanoparticles in Ginger. As shown in Figure 1A, we extracted the GELNs from ginger using the differential centrifugation method combined with an extraction kit. TEM images of the GELNs displayed spherical particles similarly with typical cup-shaped membrane structures, akin to the recognized characteristics of exosomes in the previous report (Figure 1B).[33] The result showed the GELNs with desirable particle sizes (156 ± 36 nm) (Figure 1C,D) and a negative surface charge (−26.6 ± 5 mV). Additionally, the exosomes from ginger displayed 2391.43 ± 750 ng/μL ($n = 3$) of protein concentration in this work.

Small RNA Sequencing Results. According to our sequencing results, there were 39,099,048 raw reads (Table 1). Each library obtained 14−23 million clean reads for further analysis after the removal of impurities and low-quality reads (Table 1). The size distribution of sequence lengths is summarized in Figure 2. As expected, the reads were 18−25 nt mainly, which was in accordance with the length distribution of general plant small RNAs.[34,35]

Identification of Known and Novel miRNAs. Due to the lack of genomic information on ginger, we mapped all of the filtered reads to the ginger transcriptome. The results showed that 13,650,698 sequences could be posited to the reference sequence, of which the ginger and GELN libraries account for 50.88 and 86.47%, respectively (Table S1, Supporting Information). Then, these reads were mapped onto known mature miRNAs in the miRbase. A total of 185 and 116 known miRNAs were identified separately in ginger and GELN small RNA libraries (Table S2, Supporting Information). Compared with ginger, the GELNs had 50 differentially expressed miRNAs, including 21 upregulated and 29 downregulated (Table S3, Figures 3B, and 4). Particularly, among these upregulated expressed miRNAs, the expression of 3 miRNAs (bdi-miR5179, csi-miR396e-5p, ptc-miR396g-5p)

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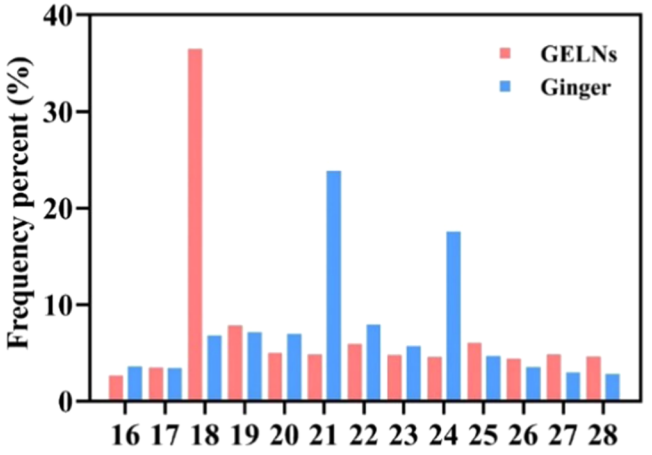

Figure 2. Length distribution of clean reads of ginger and GELN small RNA libraries.

was identified with ≥4-fold change. In addition, we found 35 and 18 novel miRNAs from the ginger and GELN libraries using miREvo and miRDeep2 software, respectively (Table S2). Similarly, six upregulated and three downregulated miRNAs were identified upon comparing the GELNs with ginger (Table S4, Figures 3B, and 4). Notably, there were three specifically expressed novel miRNAs in GELNs: novel_18, novel_20, and novel_27 (Table S4). Overall, there are 48.9% common miRNAs in the two samples and 7.7% miRNAs only exit in GELNs (Figure 3A). Since our purpose tends to study the physiological functions of miRNAs in GELNs, the subsequent target gene prediction and functional analysis were only conducted for all upregulated miRNAs (UPMs) in GELNs, compared with ginger. Figure 5 shows the fold change of expression levels for all UPMs between the GELNs and ginger.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) Validation of the miRNAs. To verify the deep sequencing data, six differentially expressed miRNAs were selected to examine their content in ginger and GELNs by RT-qPCR (Figure 6). As expected, the expression levels of three upregulated miRNAs were higher in GELNs, and the three downregulated miRNAs showed opposite expression patterns. The changing trend of these miRNAs in the above results was similar to the data obtained on the Illumina sequencing platform, indicating that our sequencing result was reliable and available.

Functional Analysis of Target Genes of the UPMs. miRNAs regulated gene expression by interacting with target mRNAs at specific sites due to inducing cleavage of the messenger RNA or inhibiting translation. To gain a better functional understanding of the miRNAs contained in GELNs, we predicted the target genes of all UPMs in the human genome by Miranda software and proceeded with the Gene Ontology (GO) and KEGG annotation. The GO annotation system analysis showed that the putative targeted genes were associated with various functions. On the cellular component, the predicted targets were mainly associated with the cell, cell part, and intracellular and intracellular part. In the molecular function category, these targets were primarily related to binding and catalytic activity. Further, the GO term biological process was enriched with targets for cellular processes and various metabolic processes, including cellular macromolecule metabolic processes and organic substance metabolic processes (Figure 7). KEGG pathway analysis is shown in Figure 8. The top 20 pathways related to target genes were significantly enriched in cancer-related pathways, endocytosis, focal adhesion, Ras signal pathways, and TNF-signaling pathways. The result implied that miRNAs contained in the GELNs possess immunomodulatory or metabolic regulation capacities.

Uptake of GELNs by Caco-2 Cells. To monitor the dynamic process of the GELN absorption, Caco-2 cells were exposed to PKH26-labeled exosomes with red fluorescence for a different duration. As shown in Figure 9A, there was no red fluorescence inside the cells in 0.25 h, after 6 h of incubation, a few red spots were centered around the blue nucleus of the Caco-2 cell, indicating that the GELNs appear to be internalized into the Caco-2 cells and transported to the place near the nucleus (Figure 9B). Further, we investigated the possible mechanism of internalizing GELNs by Caco-2 cells. Confocal analysis showed that the number of red vesicles decreased obviously in the Caco-2 cells, which were pretreated with indomethacin (Figure 10A,B). The phenomenon suggested that the GELNs were internalized via caveolin-mediated endocytosis. Amiloride is the macropinocytosis inhibitor, and the uptake of the GELNs was inhibited by 20.48 ± 14.64%. However, chlorpromazine treatment had little effect on the GELNs entering the Caco-2 cells, suggesting that clathrin-mediated endocytosis was not involved in the absorption of the GELNs. The results showed that the GELNs could be absorbed into the small intestine cells, mediated by macropinocytosis and caveolin-mediated endocytosis. The mechanism of the GELNs in the small intestine may

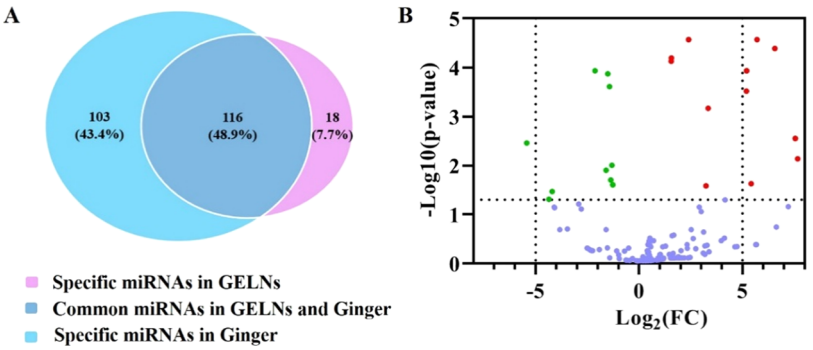

Figure 3. (A) Venn diagram of known miRNAs and novel miRNAs in ginger and GELNs. (B) Volcano plot of the different expression patterns between ginger and GELNs. Note: green solid circle, downregulated miRNAs (GELN vs ginger); red solid circle, upregulated miRNAs (GELN vs ginger); pink solid circle, miRNAs of similar expression levels between ginger and GELNs.

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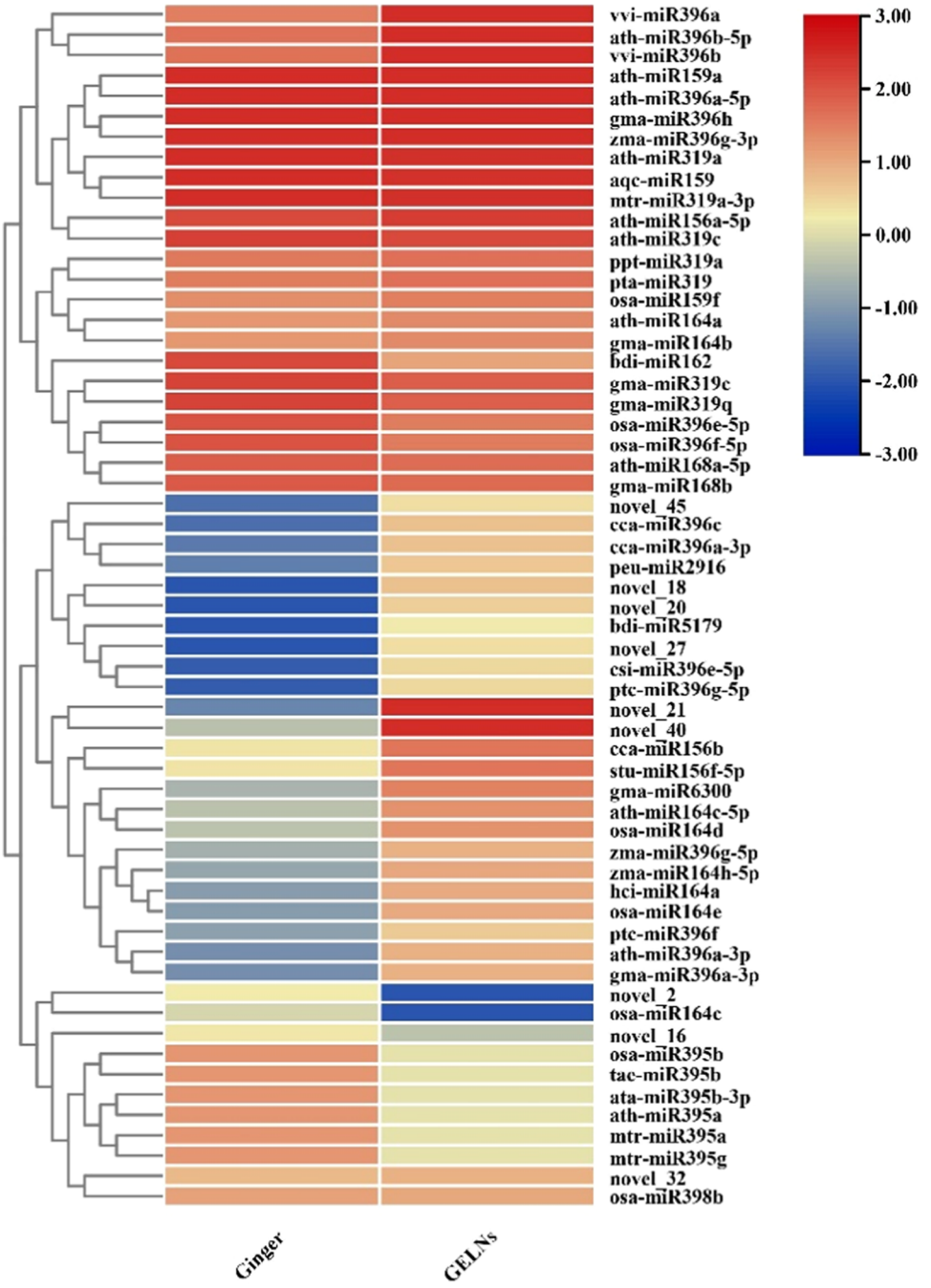

Figure 4. Heat map of differential miRNAs between ginger and GELNs. The heat map is based on the TPM analysis. Blue color represents downregulation, and red represents upregulation.

be related to the number of surface-binding proteins of the recipient cell or the characteristics of the exosome.[36] Although not experimentally validated, the advantages of delivering gene expression regulators via oral GELNs are apparent, such as efficient batch internalizing by the intestinal epithelium (Caco-2 cells) and carrying RNA for higher local concentration to better potency.

Effect of GELNs on Inflammatory Response in the LPS-Challenged Intestine Cells. We evaluated the effect of GELN administration on the expression levels of the inflammatory-related genes in the LPS-challenged intestine cell model (Figure 11). As shown in Figure 11, LPS treatment dramatically increased the mRNA levels of NF-κB, TNF-α, IL-8, and IL-1β, especially TNF-α, compared to the control group. However, the GELN treatment notably inhibited the expression of NF-κB and an array of inflammatory cytokines expression. Furthermore, to understand whether the anti-inflammatory function of GELNs was related to the miRNAs, we also investigated the effects of D-GELNs (RNA-depleted GELNs) on LPS-induced inflammation intestine cells. According to the results, the D-GELNs only showed a certain inhibitory effect on the LPS-induced TNF-α gene but did not affect the expression of other inflammatory-related genes. In general, GELNs provide a natural means of genetic material transfer to the human intestine and may promote the

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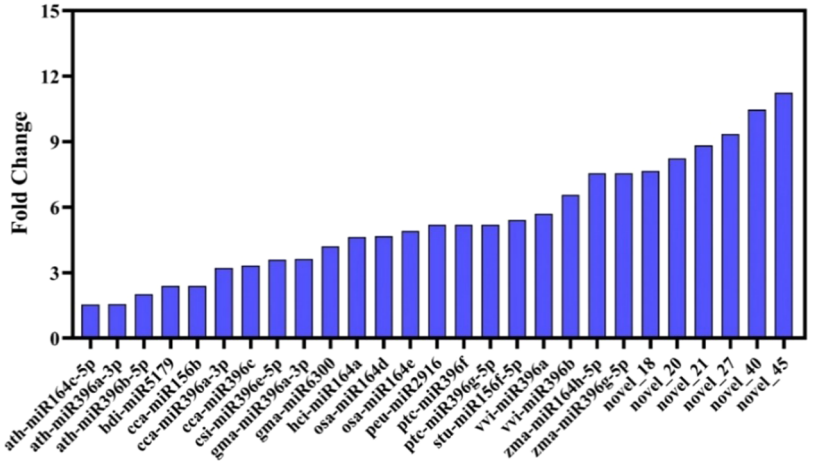

Figure 5. Comparison of the UPM expression in ginger and GELNs (GELNs vs ginger).

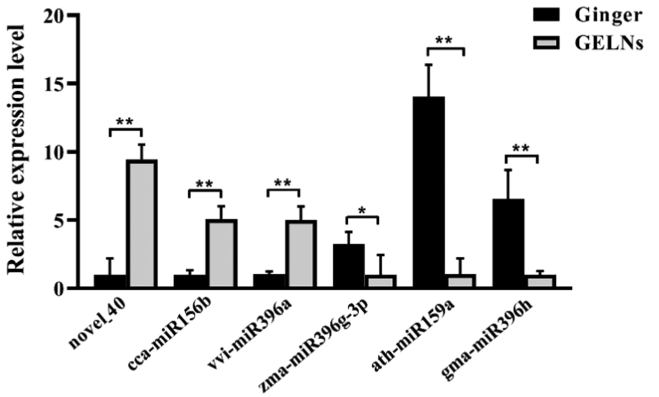

Figure 6. Expression profiles of six miRNAs in ginger and GELNs.

improvement of intestinal inflammation through cross-kingdom regulation of miRNAs.

DISCUSSION

Differential ultracentrifugation plus sucrose density gradient centrifugation is the most classical and commonly used method for the isolation of plant-derived edible nanoparticles. Although some scholars purified the exosome-like nanoparticles from sunflower seeds via selective extraction of apoplastic fluid by vacuum infiltration procedure.[37] Others extracted GELNs using PEG6000 based on the principle of polymer precipitation.[38] However, there is still a lack of available extraction methods at present. In this work, we attempted to extract GELNs by differential centrifugation combined with the commercial kit, and the feasibility of this method was confirmed via characterization of GELNs by TEM and dynamic light scattering (DLS). The GELNs were approximately intact with desirable particle sizes and a negative surface charge. This new method will provide a simple and universal strategy to purify PELNs, which will contribute to the extensive study of PELNs.

Since lin-4 was first discovered in Caenorhabditis Elegans, miRNAs have been confirmed to exist in various body fluids and exosomes.[39,40] Previous studies have indicated a total of 418 miRNAs in 11 edible plant-derived exosome-like nanoparticles, and they have unique distributions in different EPDELNs.[41] Similarly, Zhang et al. found 125 miRNAs in GELNs by deep sequencing and each contained between 15

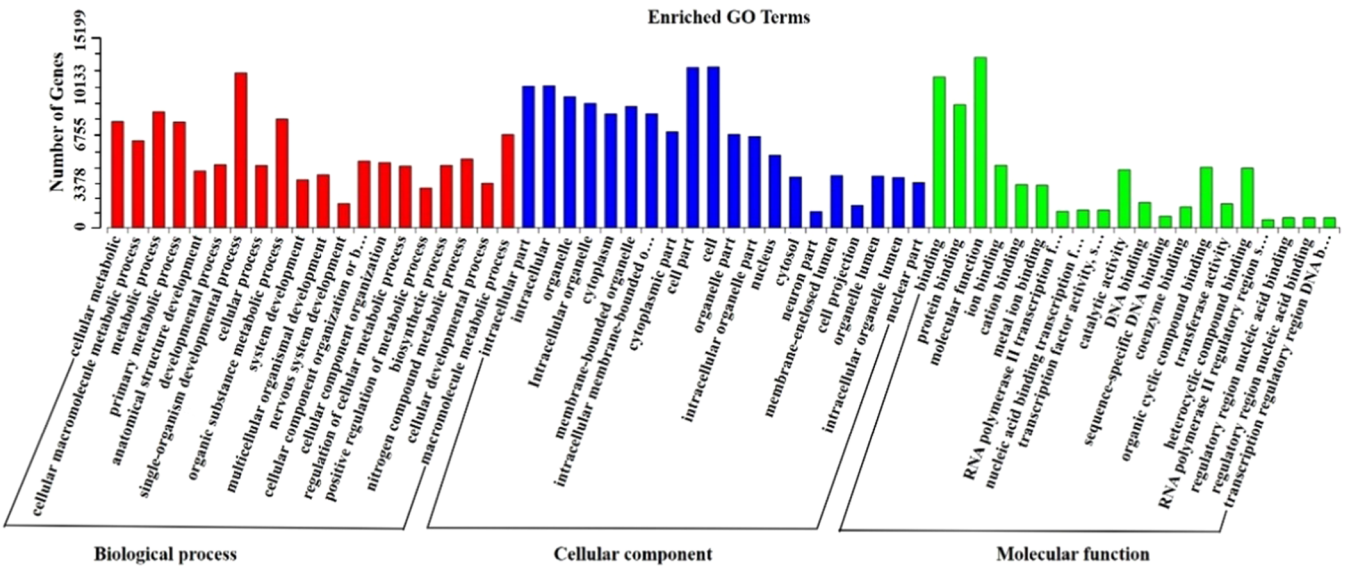

Figure 7. Gene Ontology (GO) classification of the UPMs in the GELNs.

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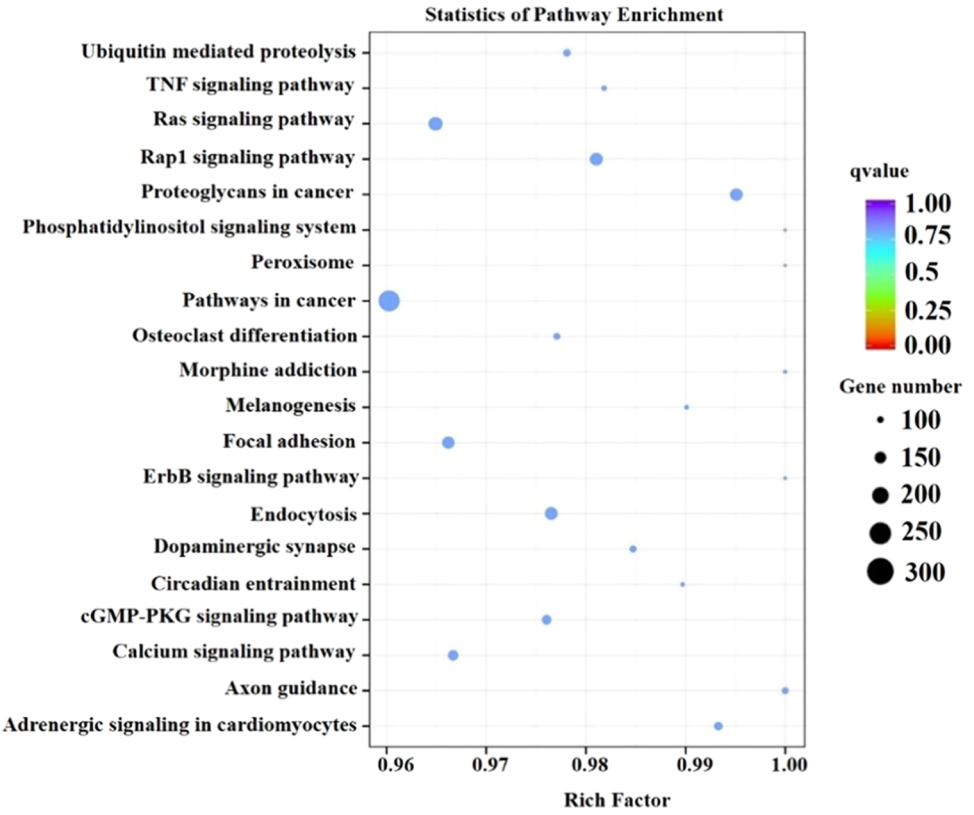

Figure 8. KEGG enrichment analysis based on the UPMs in the GELNs.

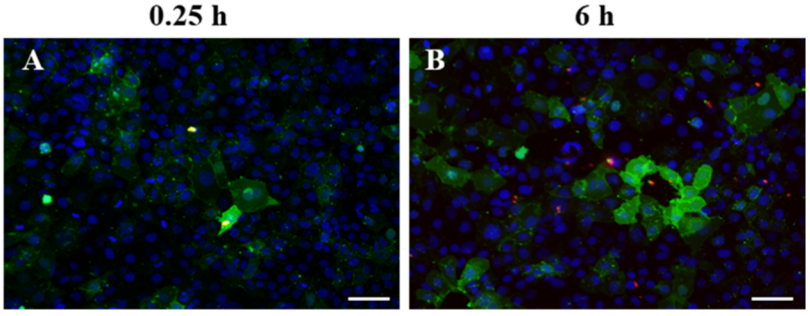

Figure 9. Fluorescence microscopy images of Caco-2 cells incubated with the PKH26-labeled GELNs for 0.25 h (A) and 6 h (B). The blue channel image shows the nuclei of Caco-2 cells stained by DAPI (blue), the red channel image shows the PKH26-labeled GELNs (red), and the green channel image shows the membrane of Caco-2 cells stained by DIO (green). The results are imaged at a 200× magnification. Scale bars represent 50 μm.

and 27 nucleotides.[11] A recent study indicated that 109 conserved miRNAs were identified in GELNs.[21] Obviously, this may be caused by many factors, including variations in plant species and cultivars, the quality of exosomes, and the processing methods of sequencing data. Although these research studies showed the miRNA profiles in various PELNs, the information of novel miRNAs and the composition difference of miRNAs between PELN and their raw material remain to be illuminated. In the study, we identified 198 miRNAs and 39 novel miRNAs in ginger and GELNs (136 from GELNs). Most co-expressed miRNAs between ginger and GELNs are involved in regulating plants' stress responses, root development, and other physiological processes. For example, miR156 in rice was identified as being related to the cold stress response. Transgenic rice with overexpressed OsmiR156k showed a lower survival rate, proline content, and chlorophyll II under cold stress.[42] Meanwhile, we noted 59 differentially expressed genes, especially 27 miRNAs (UPMs), which are highly expressed in the GELNs compared to ginger. A recent research reports that the content of plant miR159, enriched in Women's sera extracellular vesicles, was contrariwise associated with the progression of breast cancers and morbidity.[43] Another study found that the miRNA with higher enrichment in ginger ENPs, gma-miR-3600, could target the SARS-CoV-2 genome.[44] As expected, we also identified these functional miRNAs in the GELNs. Moreover, all of the UPMs could target human genes and involve a variety of immune- and metabolic-related biological pathways.

It is well established that natural plant ingredients, including PELNs, have a great influence on mammalian cell homeostasis in the digestive system. Mu et al. indicated that exosome-like nanoparticles from four edible plants (i.e., grape, grapefruit, ginger, and carrot) were taken up by intestinal macrophages and stem cells. Different edible plant-derived exosome-like

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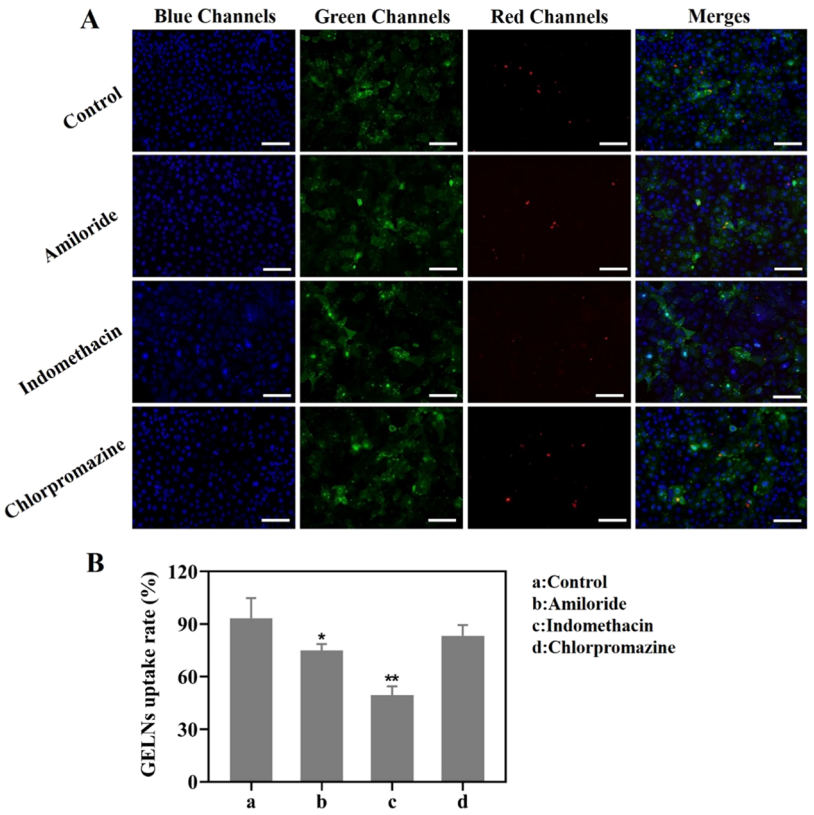

Figure 10. (A) Caco-2 cells are pretreated with amiloride, chlorpromazine, and indomethacin for 30 min and then incubated with PKH26-labeled GELNs for 6 h. Caco-2 cell nuclei were labeled with DAPI (blue); the Caco-2 cell membrane was labeled with DIO (green); and GELNs were labeled with PKH26 (red). Original magnification was 200×. (B) The percentage of GELNs uptake relative to control (n = 5).

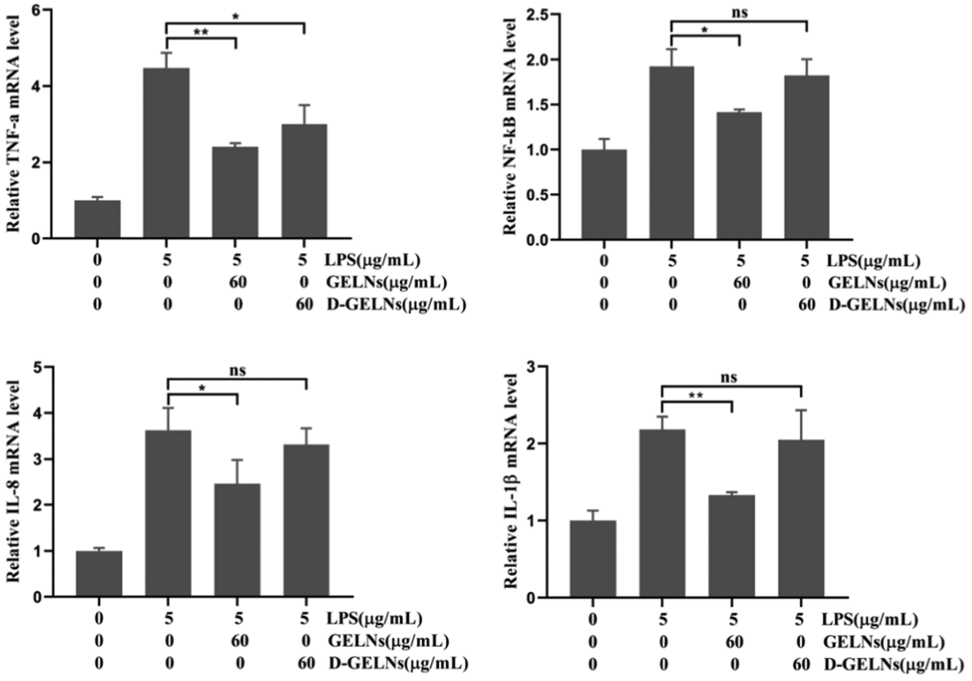

Figure 11. Effect of GELNs on the expression of genes related to inflammation.

nanoparticles have distinct biological effects on the induction of expression of anti-inflammatory genes, antioxidation gene, and activation of the Wnt/TCF4 signaling pathway.[25] The possibility that certain miRNAs may help diminish the inflammatory process allows the development of future therapies. In our present study, we showed that GELNs could be gradually internalized by human intestinal epithelial cells via macropinocytosis and caveolin-mediated endocytosis and offer protective effects against LPS-induced inflammation. Moreover, we confirmed that there is a certain correlation

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between miRNAs in GELNs and their anti-inflammatory function.

In general, this study proposed an effective method to purify the GELNs with desirable particle sizes and negative surface charges. The miRNA profiles in ginger and GELNs were analyzed using high-throughput sequencing, and the highly expressed miRNAs in the GELNs were predicted to be involved in the regulation of inflammatory and cancer-related pathways. In addition, the GELNs-containing miRNAs could be specifically internalized by the intestine cells and inhibit LPS-induced inflammation response. These results contribute to an increased understanding of the roles of miRNAs in GELNs and provide a theoretical basis for further exploration of the cross-kingdom regulation function of plant-derived genes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c07306.

Sequencing results of ginger and GELNs mapped to the ginger transcriptome; known and novel miRNAs in ginger and GELN small RNA libraries; profiles of differentially expressed known miRNAs and novel miRNAs between ginger and GELNs; and target genes of the specifically expressed miRNAs in GELNs (ZIP)

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Notes

The authors declare no competing financial interest.

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