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SHORT COMMUNICATION

# Assessment of bovine milk exosome preparation and lyophilized powder stability

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**Abstract**

Exosomes are cell-derived nanovesicles that play a crucial role in intercellular communication, presenting promising potential as biomarkers and therapeutic agents. Bovine milk exosomes (MK-Exo) show production scalability and cost-effectiveness, offering distinct advantages over cell-derived exosomes. However, exosome storage and transportation are challenging owing to their unstable nature, necessitating preservation at ultralow temperatures. Research findings suggest that freeze-drying could provide a viable solution; however, different sources of exosomes may require specific protocols. In this study, we aimed to successfully isolate high-purity MK-Exo and develop a specialized freeze-drying and lyophilization method for improved long-term preservation of MK-Exo. Specifically, the stability of the lyophilized MK-Exo was evaluated using storage stability tests. Notably, lyophilized MK-Exo remained stable for at least 3 months under high temperature of 50°C and for at least 24 months under low temperatures of 2°C–8°C, preserving their physicochemical properties and biological activity. Conclusively, these findings provide a potential solution for ambient-temperature transportation of MK-Exo, facilitating their industrial-scale production.

**KEYWORDS**

ambient-temperature storage, biological activity, bovine milk exosomes, lyophilization, stability study

## 1 | INTRODUCTION

Exosomes are a subset of extracellular vesicles (EVs), typically ranging from 40 to 150 nm in size (Kalluri, 2016). Exosomes derived from cow milk, referred to as MK-Exo (bovine milk exosomes [MK-Exo]) (Munagala et al., 2016), contain active substances (Chen et al., 2010; Hata et al., 2010; Zeng et al., 2019) and have potential applications in the food, cosmetic, nutraceutical and pharmaceutical industries. MK-Exo can penetrate the intestinal barrier (Hata et al., 2010; Rani et al., 2017; Wolf et al., 2015) and perform important functions, such as immunoregulatory, anti-bacterial and anti-oxidative activities (Zhong et al., 2023). These attributes suggest their potential utilization in the development of functional foods and their beneficial impact on diverse aspects of human health, including intestinal health, bone/muscle metabolism and microbiota regulation (Suharta et al., 2021). Moreover, MK-Exo supports intercellular adhesion and can penetrate mouse skin, indicating their potential for skin barrier penetration and repair (Wei et al., 2022) and cutaneous medical aesthetics (Xiong et al., 2021). Furthermore, encapsulation into

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exosomes enhanced the tissue delivery and biological effects of curcumin (CUR) and resveratrol (RSV) (González-Sarrías et al., 2022), demonstrates the potential application of exosomes in the nutrition industry. Notably, MK-Exo have been successfully loaded with mRNA (Zhang et al., 2023), paclitaxel (Agrawal et al., 2017) and doxorubicin (Pullan et al., 2022), confirming the drug delivery capabilities and potential application of MK-Exo in the pharmaceutical industry (Del Pozo-Acebo et al., 2021). MK-Exo, sourced from bovine milk, are considered food-safe, indicating their potential application in various consumables, cosmetics and oral medications at appropriate doses. Importantly, several studies have confirmed the non-toxic nature of MK-Exo (Agrawal et al., 2017; Munagala et al., 2016; Somiya et al., 2018). Moreover, the results of our in vivo safety evaluation tests further confirmed the safety of MK-Exo (Lu et al., 2023).

Exosomes are commonly sourced from biofluids or cell culture media supernatants. Among these sources, bovine milk offers the advantages of cost-effectiveness and scalability (Haug et al., 2007). Global bovine milk production is extensive and relatively economical, with the estimated worldwide production volume expected to reach approximately 544 million metric tons by 2022 (Statista). In 2022, the cost of milk was approximately $0.4067 per litre in New Zealand, and approximately $0.8138 per litre in Japan (CLAL). In comparison, the price per litre of cell culture medium ranges from a few hundred to thousands of dollars (Scientific; Technologies). Overall, isolating exosomes from bovine milk saves both time and expenses associated with cell culture and alleviates concerns about upstream cell culture risks.

These findings regarding functionality, safety, and potential applications of MK-Exo indicate their industrial value. However, significant challenges persist, particularly in achieving large-scale production, preservation and efficient transportation. Size-exclusion chromatography (SEC) and tangential flow filtration (TFF) have evolved as efficient isolation methods for the large-scale production of exosomes (Blans et al., 2017; Gao et al., 2022; Guo et al., 2021; Han et al., 2021; Kim et al., 2021). However, urgent attention is required to resolve the issue of preservation and transportation to facilitate industrial-scale production.

A conventional and efficient approach to store exosomes involves freezing at -80°C (Jeyaram and Jay, 2017; Thery et al., 2018; Welch et al., 2017; Witwer et al., 2013; Zhou et al., 2006). Research evidence suggests that it is feasible to store exosomes at -80°C without causing significant changes in the physical and biological properties (Agrawal et al., 2017; Lorincz et al., 2014; Munagala et al., 2016). However, this mode of storage presents limitations during transportation owing to the inconvenience and high costs associated with using dry ice (Gil et al., 2014; Roy and Gupta, 2004). Consequently, there is an urgent need for a simpler, convenient and cost-effective storage method.

Lyophilization, a technique utilized for preserving diverse biological materials, streamlines their storage and transportation without the need for laborious and expensive cold chains. Various lyophilization methods have been employed to enhance the long-term stability of exosomes. For instance, Geeurickx et al. reported that lyophilization did not impact the morphology, fluorescence intensity, number, size distribution, and density of recombinant HEK293T cell–derived exosomes (Geeurickx et al., 2019). Le Saux et al. (2020) reported that the structure of murine mesenchymal stem cell-derived EVs remained unaffected following lyophilization. Charoenviriyakul et al. (2018) found that B16BL6-derived exosomes could be stored stably at room temperature for 4 weeks after lyophilization without causing protein and RNA alterations. Additionally, Trenkenschuh et al. (2022) reported that lyophilized mammalian EVs derived from B lymphoblastoid cells retained their original particle size and concentration without cargo loss when stored at 2°C–8°C for 1 month. Currently, research on exosome lyophilization lacks systematic and comprehensive investigation, and data on the stability of lyophilized MK-Exo, especially under long-term preservation, are limited.

In this study, we aimed to successfully isolate high-purity MK-Exo and develop a specialized freeze-drying and lyophilization method for improved long-term preservation of MK-Exo. Considering the variations in lyophilization methods for exosomes derived from different sources, we developed a specialized lyophilization method for MK-Exo. Additionally, stability experiments were performed to examine the long-term stability of lyophilized MK-Exo.

## 2 | MATERIALS AND METHODS

### 2.1 | Preparation of MK-Exo using a TFF-SEC based protocol

The milk pH was adjusted to pH 4.6 using hydrochloric acid (Merck, Billerica, MA, USA), followed by centrifugation at 4000 *g* (Beckman) for 30 min at 4°C. The supernatant was collected and filtered using depth filtration (3 M, Minnesota, USA). The filtered supernatant was concentrated using TFF (100 KD, Repligen, Massachusetts, US) and the PB buffer (pH, 4.6) was replaced with PBS. Thereafter, the ultrafiltered sample (casein-free whey) was fractionated using SEC for exosome purification (Packing model 4FF, Huiyan Bio, Wuhan, China), and the sample of the external water peak was collected, which is the MK-Exo. Finally, the resulting MK-Exo were sterile-filtered using a 0.22-μm filter and stored at -80°C until further use.

## 2.2 | Preparation of MK-Exo using density-gradient ultracentrifugation (DC)

Briefly, 40 mL of casein-free whey was subjected to sucrose density gradient centrifugation as previously described (Hata et al., 2010). Thereafter, the casein-free whey was centrifuged at 100,000 *g* (Beckman Coulter, USA) for 105 min at 4°C, and the resultant pellet was resuspended in 2 mL of 250 mM Tris-HCl solution (pH 7.4). The resuspended pellet was subjected to the top of a discontinuous density gradient consisting of 2 M (1.309 g/mL), 1.65 M (1.141 g/mL), 1.3 M (1.107 g/mL), 0.95 M (1.084 g/mL) and 0.6 M (1.057 g/mL) sucrose (7 mL volume for each angle) in 250 mM Tris-HCl solution. Thereafter, the pellets were centrifuged at 100,000 *g* at 4°C for 20 h, and the exosomal fraction between fractions 3 (1.3 M) and 4 (1.65 M) was collected. To remove sucrose, the fraction was diluted in PBS to a final volume of 40 mL and centrifuged at 100,000 *g* for 105 min at 4°C. The pellet was resuspended in 1 mL of PBS. Finally, the DC-prepared MK-Exo were sterile-filtered using a 0.22-μm filter and stored at -80°C until further use.

## 2.3 | Transmission electron microscopy (TEM)

Briefly, MK-Exo (100 μg/mL) was fixed in 2% (w/v) paraformaldehyde for 15 min. Thereafter, a 10-μL mixture was mounted on a formvar-carbon-coated grid (Beijing XXBR Technology, Beijing, China) for 3 min, followed by staining with a uranyl oxalate solution (4% w/v uranyl acetate and 0.0075 M oxalic acid, pH 7) for 1 min. Finally, the samples were observed using a TEM (Hitachi High-Technologies Corporation, Tokyo, Japan).

## 2.4 | Particle size distribution and number analysis

The particle size and number of MK-Exo were characterized using a NanoFCM instrument (NanoFCM Inc., Xiamen, China) according to the manufacturer's instructions. A silica nanosphere cocktail (cat. S16M-Exo, NanoFCM Inc.) containing a mixture of 68, 91, 113 and 155 nm standard beads was used to adjust the instrument for particle size measurement. The instrumental parameters were set as follows: laser, 10 mW, 488 nm; SS decay, 10%; sampling pressure, 1.0 kPa; sampling period, 100 μs; and time to record, 1 min.

## 2.5 | Purity analysis of MK-Exo

Briefly, the purity of MK-Exo was analysed using a SEC-1000 column (7.8 mm × 150 mm, 7 μm; Thermo Fisher Scientific) coupled with UPLC H-Class system (Waters). The column was eluted at a flow rate of 0.3 mL/min using 150 mM NaCl and 20 mM phosphate buffer (pH 7.2). The UV absorbance was measured at a wavelength of 280 nm.

## 2.6 | Zeta potential measurement

The Zeta potential of MK-Exo was measured thrice at 25°C using the following parameters: sensitivity, 85; shutter value, 70; and frame rate, 30 frames per second. Data were collected and analysed using the Brookhaven ZetaPALS software (Midekessa et al., 2020).

## 2.7 | Quantitative assessment of protein concentration

The protein concentration of MK-Exo was measured using a BCA Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

## 2.8 | Proteomic analysis

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was performed using an Easy NLC 1200-Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific). The nano-HPLC system was equipped with an Acclaim PepMap nano-trap column (C18, 100 Å, 75 μm × 2 cm) and an Acclaim Pepmap RSLC analytical column (C18, 100 Å, 75 μm × 25 cm). For this study, 1 μL (20 μg) of the peptide mix was loaded into the enrichment (trap) column. All spectra were acquired in positive mode, using

**TABLE 1** The lyophilization procedure.

| Temperature (°C) | Vacuum (pa) | Time (h) |
|---|---|---|
| −50 | / | 1 |
| −20 | / | 1 |
| −50 | / | 1 |
| −50 | 20 | 1 |
| −40 | 20 | 1 |
| −30 | 20 | 1 |
| −25 | 20 | 24 |
| 20 | 20 | 15 |

full-scan MS spectra scanning in the FT mode from m/z 300–1650 at a resolution of 70,000. For MSMS, the 15 most intense ions with charge states ≥ 2 were isolated within an isolation window of 1.6 m/z and fragmented through higher-energy collisional dissociation (HCD) with a normalized collision energy of 28. A dynamic exclusion time of 30 s was used.

Raw files were searched using Proteome Discoverer (version 2.4; Thermo Fisher Scientific) employing Sequest as the search engine. The fragment and peptide mass tolerances were set at 20 mDa and 10 ppm, respectively, allowing for a maximum of two missed cleavage sites. A false discovery rate (FDR) of 1% was employed for proteins and peptides. Furthermore, differentially expressed proteins (DEPs) were analysed to identify the most significantly enriched signal transduction pathways using DAVID Bioinformatics Resource 2021 (http://david.abcc.ncifcrf.gov/).

## 2.9 | Determination of contaminant milk proteins in MK-Exo preparations

Briefly, whole milk protein and β-lactoglobulin contents of MK-Exo were measured using the RIDASCREEN FAST Milk Kit (R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's instructions. Casein content was measured using the PriboFast Casein (Bovine milk) ELISA Kit (Pribolab, Qingdao, China). Additionally, the IgM and IgG levels were determined using the Strongyloides IgG/IgM ELISA Kit (Abcam, Cambridge, UK).

## 2.10 | Lyophilization of MK-Exo

MK-Exo were lyophilized using previously established exosome lyophilization methods (Geeurickx et al., 2019; Hongchao et al., 2018). Specifically, we developed a specific method for lyophilization using trehalose and mannitol as cryoprotectants. Before mixing with purified MK-Exo, trehalose and mannitol were prepared into a solution of a certain concentration using PBS. Notably, the final concentration of trehalose and mannitol was 5% (w/v), while the final concentration of MK-Exo was less than 300 μg/mL. Typically, 1 mL of the resulting exosome suspension was transferred to 2-mL cryogenic vials and lyophilized (SCIENTZ, Ningbo, China) following the procedure in Table 1.

## 2.11 | Determination of the water content

The water content of the lyophilized MK-Exo powder was measured at least twice using the KEM MKA-610 Karl-Fischer Moisture Titrator (Kyoto Electronics, Kyoto, Japan).

## 2.12 | Treatment of Caco-2 cells with MK-Exo

The human epithelial colorectal adenocarcinoma (Caco-2) cell line was purchased from the National Institute of Cell Resources (Beijing, China). Caco-2 cells were grown in Dulbecco's modified Eagles's medium (DMEM) (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 100 μg/mL of penicillin-streptomycin. The cells were maintained in a 5% $CO_2$ humidified atmosphere at 37°C.

Caco-2 cells were seeded in 6-well plates (Corning Costar) at an initial density of 350,000 cells/well and incubated for approximately 24 h. Thereafter, the cells were incubated with 75 μg/mL of MK-Exo or lyophilized MK-Exo (Lyo MK-Exo). Cells treated with the lyophilized protective agent (5% [W/V] trehalose and mannitol in PBS) were used as controls. After incubating for 24 h, the supernatant was discarded, and the cells were washed twice with PBS, collected in RIPA Lysis Buffer (Beyotime, China), and stored at -20°C until protein extraction.

## 2.13 | Western blotting

Western blotting was performed as previously described (Lu et al., 2013). Protein concentration of MK-Exo preparations or cellular lysates was determined with a BCA protein assay kit (Thermo, A53226). Thereafter, proteins (10 μg) were separated using SDS-polyacrylamide (12%) gel and transferred to a poly-vinylidene fluoride membrane (Millipore, ISEQ00010). After blocking with 5% (W/V) bovine serum albumin (BSA) for 1 h at 25°C, the membrane was incubated with anti-GAPDH (Proteintech, 60004-1) or anti-claudin 1 (Abcam, ab211737) overnight at 4°C. The membranes were washed with Tris-buffered saline with Tween (TBST) and incubated with horseradish peroxidase (HRP) secondary antibody (dilution, 1:10,000) for 1 h at room temperature. After washing excess secondary antibody with TBST, antibody reaction was detected using the FluorChem E system (Pro-teinSimple, USA) according to the manufacturer's protocol.

## 2.14 | Expi293F and MSC cells culture

Human embryonic kidney Expi293F (Thermo Fisher Scientific) cells were cultured in Expi293F media (ExCell Bio, China) at 37°C in 8% $CO_2$ under constant agitation at 110 rpm. Cells were seeded at a density of 1.5–2 × $10^6$ cells/mL and harvested at a density of 5–6 × $10^6$ cells/ml in 2.8 L roller bottles (Duoning, shanghai, China).

Human Mesenchymal stem cells (hMSCs) were purchased from the Ambitious Biotechnology Inc. (Tianjin, China) and cultured with MSC Serum-free basal medium (Yocon, Beijing, China) for 72 h.

## 2.15 | Preparation of Expi293F cells and MSC exosomes

Expi293F cells, derived from the human embryonic kidney cell line HEK293, were cultured as previously described above (Section 2.14) and centrifuged at 300 *g* for 10 min. Thereafter, the supernatant was further centrifuged at 3000 ×*g* for 10 min at 4°C, and the resulting supernatant was harvested. Human mesenchymal stem cell supernatant was harvested and centrifuged at 300 *g* for 10 min (Wang et al., 2021). Thereafter, hMSC-exo and 293-exo were purified from the collected supernatants through density gradient centrifugation, and both the exosomal fraction between fractions 2 (0.95 M/L) and 3 (1.3 M/L) were collected.

## 2.16 | Statistical analyses

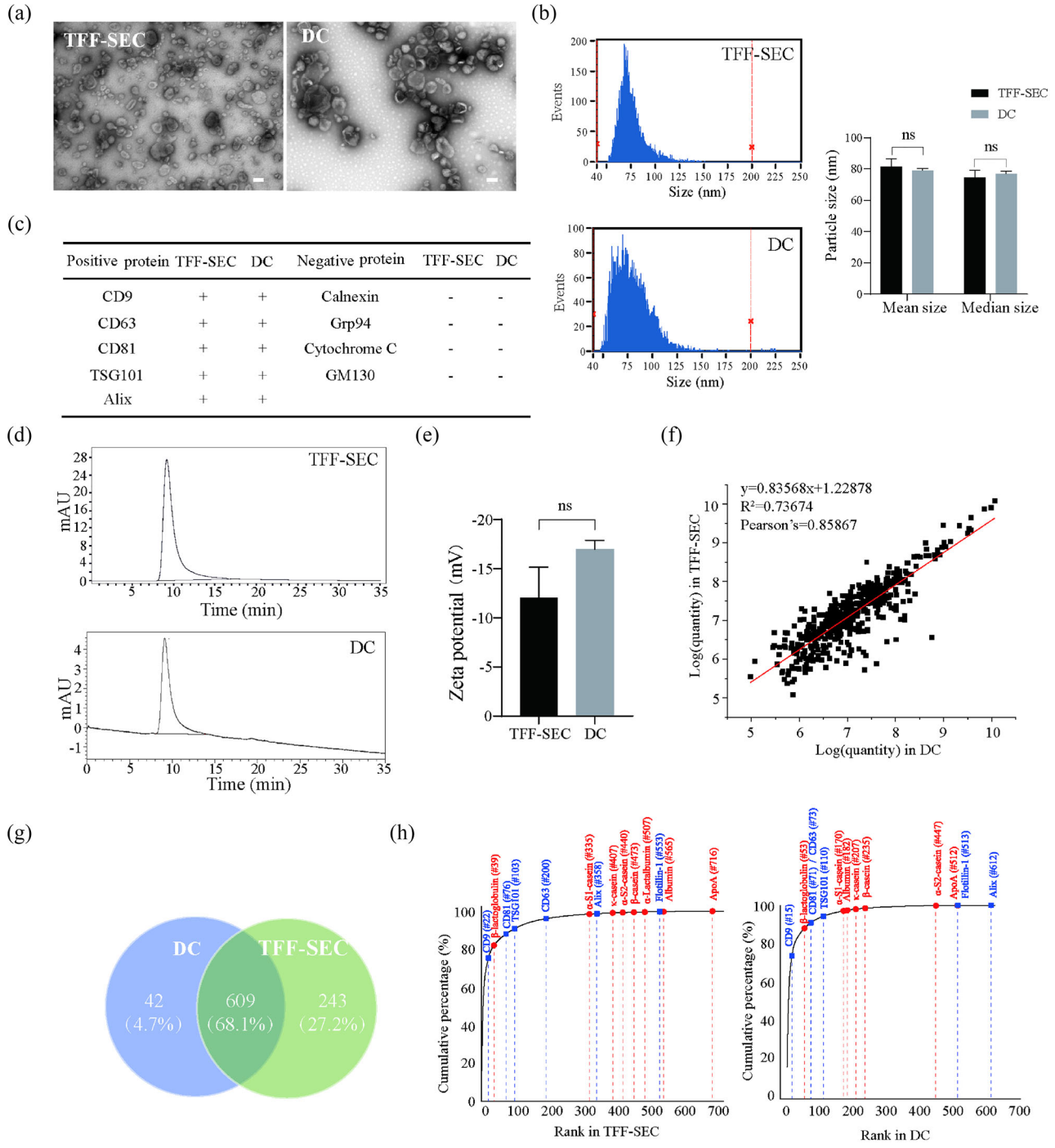
All statistical analyses were performed using the GraphPad Prism software (version 8.0; GraphPad Software, San Diego, CA, USA). Data are presented as mean ± standard deviation (SD). Protein quantity accumulation and correlation analysis were performed using the Origin software. Statistical comparisons were evaluated using Student's *t*-test. Statistical significance was set at $P < 0.05$. Venn diagrams were created using the Venn platform (http://bioinfogp.cnb.csic.es/tools/venny/index.html), and heatmaps were generated using the bioinformatics platform (https://www.bioinformatics.com.cn/).

# 3 | RESULTS

## 3.1 | Preparation and characterization of MK-Exo

To establish a good manufacturing practice (GMP) – compliant MK-Exo preparation method, we developed a method combining TFF and SEC. Overall, three batches of MK-Exo were prepared and characterized. TEM showed the presence of particles with the concave tea-tray shape (Figure 1a), a classic morphology of exosomes. Additionally, more than 98% of the particles fell within the size range of 40–150 nm (Figure 1b). Moreover, proteome analysis showed the presence of typical EV-markers (CD9, CD63, CD81, TSG101 and Alix) and the absence of the contaminant marker proteins calnexin, Grp94, cytochrome and GM130 (Figure 1c). Furthermore, the results of the western blotting are presented in Figure S1. Overall, these results confirmed the successful isolation of MK-Exo via the TFF-SEC based protocol.

Density gradient centrifugation (DC) is considered the "gold standard" for exosome isolation (Gao et al., 2022). Therefore, we compared the characteristics of MK-Exo isolated using DC and TFF-SEC methods. MK-Exo isolated using TFF-SEC and DC purification method all showed the same concave tea-tray shape, with particle size ranging from 40 to 200 nm. Additionally, both isolates expressed the same typical EV-markers (CD9, CD63, CD81, TSG101 and Alix). Overall, the comparative analysis revealed no significant differences in morphology, particle size, or protein markers between MK-Exo isolated using the two purification methods (Figure 1a–c). Furthermore, purity analysis using HPLC indicated that MK-Exo isolated using TFF-SEC




| Positive protein | TFF-SEC | DC | Negative protein | TFF-SEC | DC |
|---|---|---|---|---|---|
| CD9 | + | + | Calnexin | - | - |
| CD63 | + | + | Grp94 | - | - |
| CD81 | + | + | Cytochrome C | - | - |
| TSG101 | + | + | GM130 | - | - |
| Alix | + | + | | | |

**FIGURE 1** Characterization of bovine milk-derived exosomes (MK-Exo) prepared using tangential flow filtration combined with size exclusion chromatography (TFF-SEC) and density gradient centrifugation (DC). (a, b) Morphological characteristics and particle size analysis of MK-Exo prepared using TFF-SEC and DC methods; Scale bar = 100 nm. (c) Positive and negative marker proteins of MK-Exo. (d, e) Purity analysis and zeta potential of MK-Exo. Error bars represent the standard deviation of three repetitive experiments. "ns" indicates no significant difference. (f) Protein quantity accumulation analysis comparing specific surface proteins to major milk proteins (impurity protein) of MK-Exo. (g) Venn diagram depicting MK-Exo proteome. (h) Protein correlation analysis between the MK-Exo prepared using the TFF-SEC and DC methods.

and DC methods showed a single peak (Figure 1d), with average zeta values of −12 mV and −17 mV, respectively (Figure 1e). Collectively, these findings suggest that MK-Exo obtained using both methods have similar physicochemical properties. Proteome analysis identified and quantified 894 protein groups in MK-Exo isolated using both purification methods (Figure 1g), Notably, 68.1% (609/894) protein groups were detected in both MK-Exo. Additionally, 243 distinct protein groups were identified in MK-Exo isolated using TFF-SEC method compared with that in DC-isolated MK-Exo, indicating that more intrinsic

**TABLE 2** Detection of impurity proteins in MK-Exo isolated using density gradient centrifugation (DC) and size exclusion chromatography (SEC).

| Impurity proteins | SEC Mean ± SD | DC Mean ± SD | *P*-value |
|---|---|---|---|
| Whole milk protein (μg/mL) | 3 ± 0.22 | 21.51 ± 14.63 | 0.19 |
| casein (μg/mL) | 0.11 ± 0.07 | 2.25 ± 1.53 | 0.07 |
| Beta-lactoglobulin (μg/mL) | 0 ± 0 | 1.02 ± 0.76 | 0.0801 |
| IgM (μg/mL) | 6.29 ± 3.36 | 20.5 ± 28.2 | 0.43 |
| IgG (μg/mL) | 0.21 ± 0.09 | 0.53 ± 0.34 | 0.19 |

protein species were retained during TFF-SEC purification. Further analysis of the 609 overlapped proteins showed a significant correlation ($R^2 = 0.73674$) between the quantities of proteins in MK-Exo isolated using TFF-SEC and DC methods (Figure 1f). Additionally, we plotted cumulative curves of protein quantities for each MK-Exo proteome, and the proteins were ranked from high to low per quantity (Figure 1h), proteome data is provided in the supporting file. Typical EV-markers or contaminants were quantified in both proteomes. Notably, the typical EV-markers, including CD81, CD9, TSG101, CD63 and flotillin-1, shared a similar ranking between the TFF-SEC and DC groups. Moreover, the lower rankings of the contaminant proteins $\beta$-lactoglobulin (rank #39), $\alpha$-S1-casein (rank #335), $\kappa$-casein (rank #407), $\beta$-casein (rank #473), albumin (rank #565) and ApoA (rank #716) in TFF-SEC-isolated MK-Exo indicated a decrease in heteroprotein content in the TFF-SEC-isolated MK-Exo. Furthermore, MK-Exo isolated using the TFF-SEC method exhibited significantly lower whole-milk protein content than those isolated using the DC method. Consistent with the proteome data, the presence of other contaminant proteins (casein, $\beta$-lactoglobulin) was minimal and inconspicuous (Table 2). Collectively, these results highlight the successful isolation of high-quality MK-Exo using the TFF-SEC based method. The isolated MK-Exo was utilized for subsequent storage and stability studies.

## 3.2 | Validation of the methodology used for MK-Exo characterization

To ensure the reliability, linearity and precision of the experimental data, the methodology used for MK-Exo characterization was validated against the ICH M10 guidelines (2022). NanoFCM was selected to determine the mean and median size of the particles owing to its simplicity and convenience (Tables S1–S2). Both the result of each run (within-run) and in different runs (between-run) exhibited precision within ± 15% of the nominal concentration (Table S3–S4), meeting the requirements of methodology validation.

Additionally, LC-MS/MS was employed for the detection of protein markers (Table S5). Furthermore, we verified the accuracy, precision, dilution linearity of the protein concentration, purity, zeta potential and whole-milk protein analysis methods (Table S6–S17). Overall, these verification results affirm the accuracy and precision of our adopted analysis method. Conclusively, the data obtained from our experiments exhibit high reliability and credibility.

## 3.3 | Determination of the effect of storage at -80°C on MK-Exo

To confirm the effect of storage temperature, we stored three batches of MK-Exo suspension at -80°C (Agrawal et al., 2017; Munagala et al., 2016) for 8 months and evaluated changes in morphology, particle size and purity. Comparative analysis of the three batches before and after 8 months of storage at -80°C showed that MK-Exo maintains their concave tea-tray shape and particle size distribution of 40–200 nm, with average particle sizes of 50 and 60 nm, respectively, and median particle sizes of 50 and 60 nm, respectively. Overall, there were no significant changes in the morphology and particle size of MK-Exo after 8 months of storage at -80°C (Figure 2a,b). However, there was a significant decrease in purity (Figure 2c). Notably, purity analysis of MK-Exo revealed non-singular peaks, potentially indicating the release of proteins. Collectively, these findings indicate potential instability in the physical properties of liquid MK-Exo following prolonged storage at -80°C.

## 3.4 | Limitations for MK-Exo lyophilization for exosomes

To address the limitations of long-term storage of MK-Exo at -80°C, we employed a freeze-drying method that is commonly used for other exosomes (Geeurickx et al., 2019; Hongchao et al., 2018). Specifically, fresh MK-Exo was lyophilized using 5% or 10% (W/V) trehalose. However, the lyophilized MK-Exo samples exhibited notable issues, such as shrinkage, collapse, non-cake-like appearance and stickiness (Figure 2d), indicating that the moisture content of the TFF-SEC MK-Exo remains high

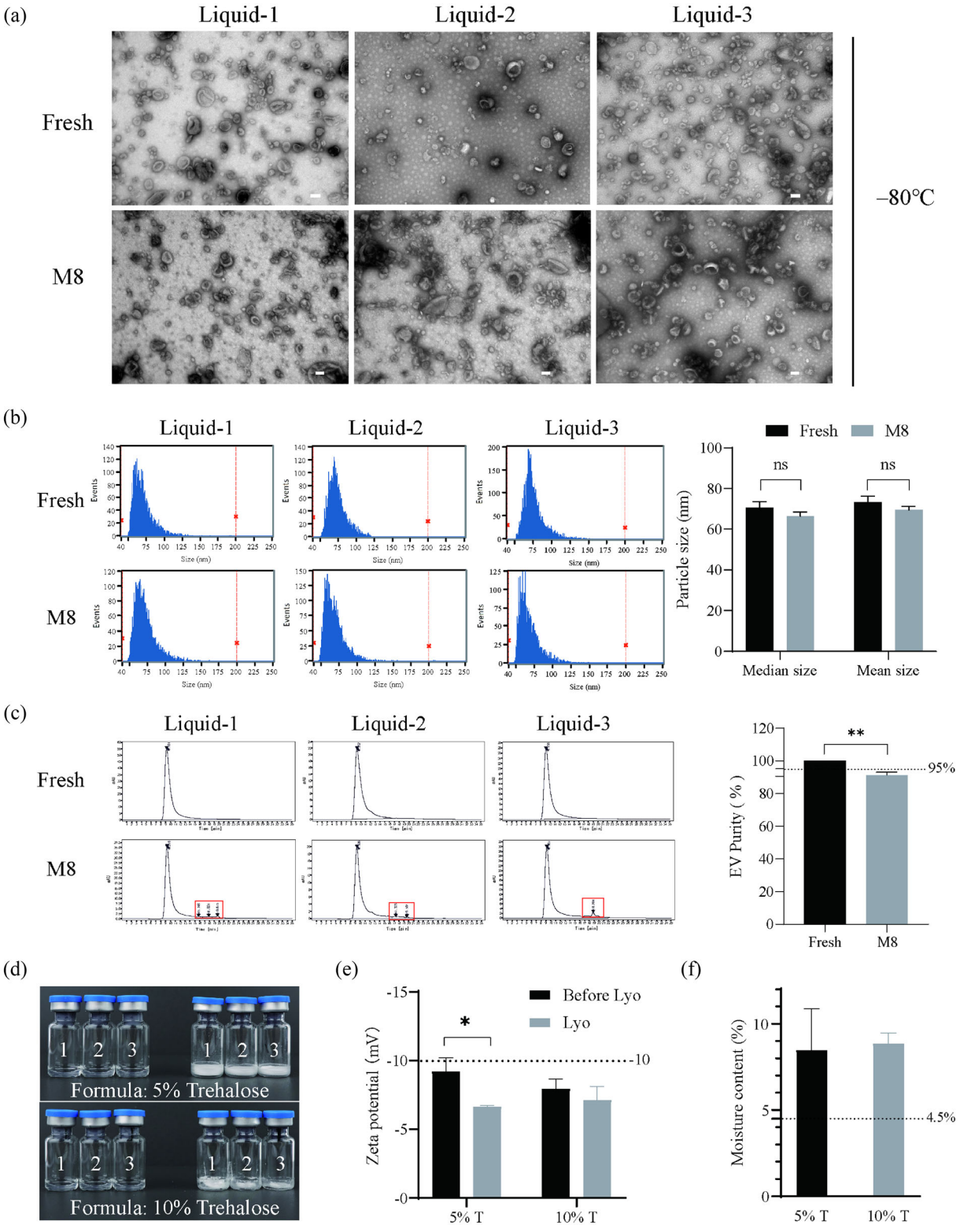



**FIGURE 2** Characterization of liquid bovine milk-derived exosomes (MK-Exo) before and after storage at -80°C. (a–c) Morphological characterization, particle size analysis, and purity analysis of liquid MK-Exo; Scale bar = 500 nm. (d) Appearance of lyophilized liquid MK-Exo (formula: 5% or 10% trehalose) prepared according to a previously described method. (e, f) Zeta potential and moisture content of MK-Exo before and after lyophilization. Error bars represent the standard deviation of three replicates. "ns" indicates no significant difference. $^{**}P < 0.01$, $^{*}P < 0.05$.

even after the freeze-drying process (Figure 2f). Additionally, there was a significant decrease in the zeta potential of MK-Exo lyophilized with 5% trehalose, whereas only a slight decrease (non-significant) was observed in MK-Exo lyophilized with 10% trehalose (Figure 2e). Overall, these results suggest that the lyophilization process damaged the MK-Exo, leading to an increase in sample instability. Additionally, lyophilization methods applied to other exosomes (HEK293T cell-derive exosomes and human adipose-derived stem cells exosomes) may not be suitable for MK-Exo. Consequently, there is a need to develop a specialized lyophilization method tailored specifically for MK-Exo.

## 3.5 | Development of a MK-Exo lyophilization method

To obtain high-quality lyophilized MK-Exo, we developed a specialized lyophilization method suitable for MK-Exo with formula trehalose and mannitol was 5% (w/v) and characterized the resulting product. Notably, lyophilized MK-Exo produced using our method exhibited a white cake-like appearance without shrinkage or collapse across three separate batches. Consistent with our quality standards, the moisture content of the lyophilized MK-Exo was less than 4.5% (Figure 3a). TEM showed that both pre- and post-lyophilized MK-Exo maintained a classic central depression shape (Figure 3b). Additionally, nanoFCM analysis showed no significant differences in particle diameter, mean size, or median size before and after lyophilization (Figure 3c). Moreover, purity analysis revealed a single peak (Figure 3d), with no significant difference in zeta potential (Figure 3e). Collectively, these findings suggest that our lyophilization method has minimal impact on the properties of MK-Exo.

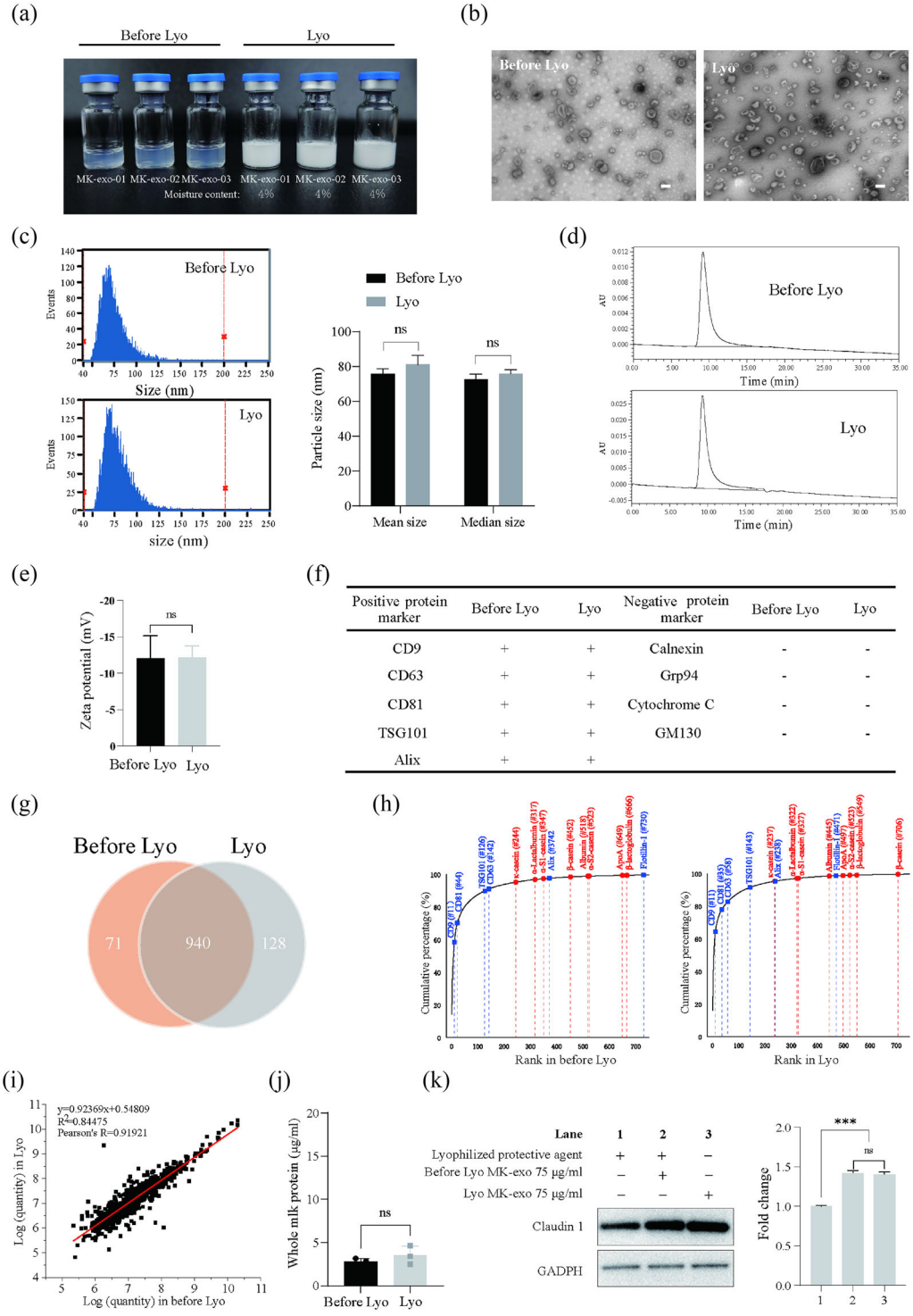
To evaluate the impact of freeze-drying on MK-Exo, we analysed the protein composition of MK-Exo before and after freeze-drying. Importantly, there were consistencies in the expression levels of the typical EV-markers (CD9, CD63, CD81, TSG101 and Alix) and contaminant marker proteins (calnexin, Grp94, cytochrome and GM130) in the MK-Exo before and after lyophilization (Figure 3f). Additionally, the results of the Western blot analysis results are shown in Figure S2. Moreover, Venn diagram showed no significant differences in protein composition between pre- and post-lyophilized MK-Exo (Figure 1g). Furthermore, the cumulative curve indicated similar protein ordering and heteroprotein content (Figure 3h), proteome data is provided in the supporting file. Importantly, the correlation curve indicated a high correlation between the proteins in the two states (Figure 3i). Consistent with the quality standards, there was no significant difference in the whole-milk protein content of the Mk-Exo before and after lyophilization, (Figure 3j). Overall, these results suggest that lyophilization using the specialized method does not induce significant changes in the protein composition of MK-Exo.

To investigate the effect of lyophilization on MK-Exo activity, we examined the activity of MK-Exo in Caco2 cells before and after lyophilization using the Western blot analysis (He et al., 2021). Notably, there was no significant difference in MK-Exo activity before and post-lyophilization as claudin-1 protein expression was upregulated in both samples (Figure 3k). Overall, these results suggest that lyophilization does not significantly affect the biological activity of MK-Exo. Therefore, it can be concluded that MK-Exo was successfully lyophilized without compromising its physicochemical properties and biological activity.

However, applying the developed MK-Exo lyophilization method to freeze-dry exosomes obtained from MSCs and Expi293 cells resulted in a significant decrease in sample purity (Figure S3a–c, d–g). Overall, this observation emphasizes that the developed lyophilization method is specific to MK-Exo and may not be applicable to other types of EVs.

## 3.6 | Stability studies of lyophilized MK-Exo under accelerated storage condition

In this study, experiments were performed to confirm the stability of freeze-dried MK-Exo prepared using a TFF-SEC based protocol at accelerated storage conditions. After re-dissolution in water, the stability of MK-Exo lyophilized samples was assessed at various time points (days 0, 14, 30, 60 and 90) and temperatures (20°C, 30°C, 40°C and 50°C) during storage (Figure 4a). Notably, significant alterations were observed in the morphology of MK-Exo after storage at 50°C for 3 months, whereas no significant changes were detected at other temperatures (Figure 4b). Additionally, there were no significant changes in the particle size of MK-Exo across all detection points during the storage period, with over 98% of the particles distributed in the range of 40–150 nm (Figure 4c and Figure S4). However, the purity of MK-Exo decreased the most when stored at 50°C, falling below the quality control standards at 3 months. Although no significant difference was observed, the HPLC profile showed two peaks. (Figure 4d and Figure S5). Furthermore, the protein concentration, zeta potential, moisture content, and whole-milk protein content exhibited no significant changes at each testing point, consistently meeting the quality control standards (Figure 4E–H). Moreover, the marker protein for MK-Exo remained consistent throughout the storage period (Table S18), and heatmap analysis revealed no significant differences in the proteome (Figure S6), proteome data is provided in the supporting file. A linear correlation analysis of the proteomic data showed a high correlation between Day 0 (D0) and different time points, indicating negligible changes in the proteome across all time points (Table 3 and Figure S7). Collectively, these findings indicate that lyophilized MK-Exo can be stored for up to 3 months below 40°C and for at least 2 months at 50°C, which makes it suitable for transportation at normal temperatures. According to the guidelines outlined in document *YY/T 0681.1-2018 Test methods for sterile medical device package*, the shelf life of lyophilized MK-Exo at a temperature of 25°C was estimated to be 339.41 days.




| Positive protein marker | Before Lyo | Lyo | Negative protein marker | Before Lyo | Lyo |
|---|---|---|---|---|---|
| CD9 | + | + | Calnexin | - | - |
| CD63 | + | + | Grp94 | - | - |
| CD81 | + | + | Cytochrome C | - | - |
| TSG101 | + | + | GM130 | - | - |
| Alix | + | + | | | |

**FIGURE 3** Lyophilized exosomes were characterized to confirm the feasibility of the method. (a) Images before lyophilization (Before Lyo) and after lyophilization (Lyo MK-Exo). (b–e) Analysis of the main morphological features, particle size, purity, and zeta potential of MK-Exo; Scale bar = 500 nm. (f) Condition of the protein markers of MK-Exo. (g) Proteome Venn diagram comparing MK-Exo before and after lyophilization. (h) Protein quantity accumulation analysis of specific exosomes surface proteins and major milk proteins (impurity proteins) of MK-Exo. (i) Protein correlation analysis of MK-Exo before and after lyophilization. (j) Assessment of whole-milk protein content in MK-Exo. Error bars represent the standard deviation of three replicates. "ns indicates no significant difference. ***$P < 0.001$. (k) Analysis of claudin-1 and GAPDH expression using the western blotting and grayscale-based band analysis.

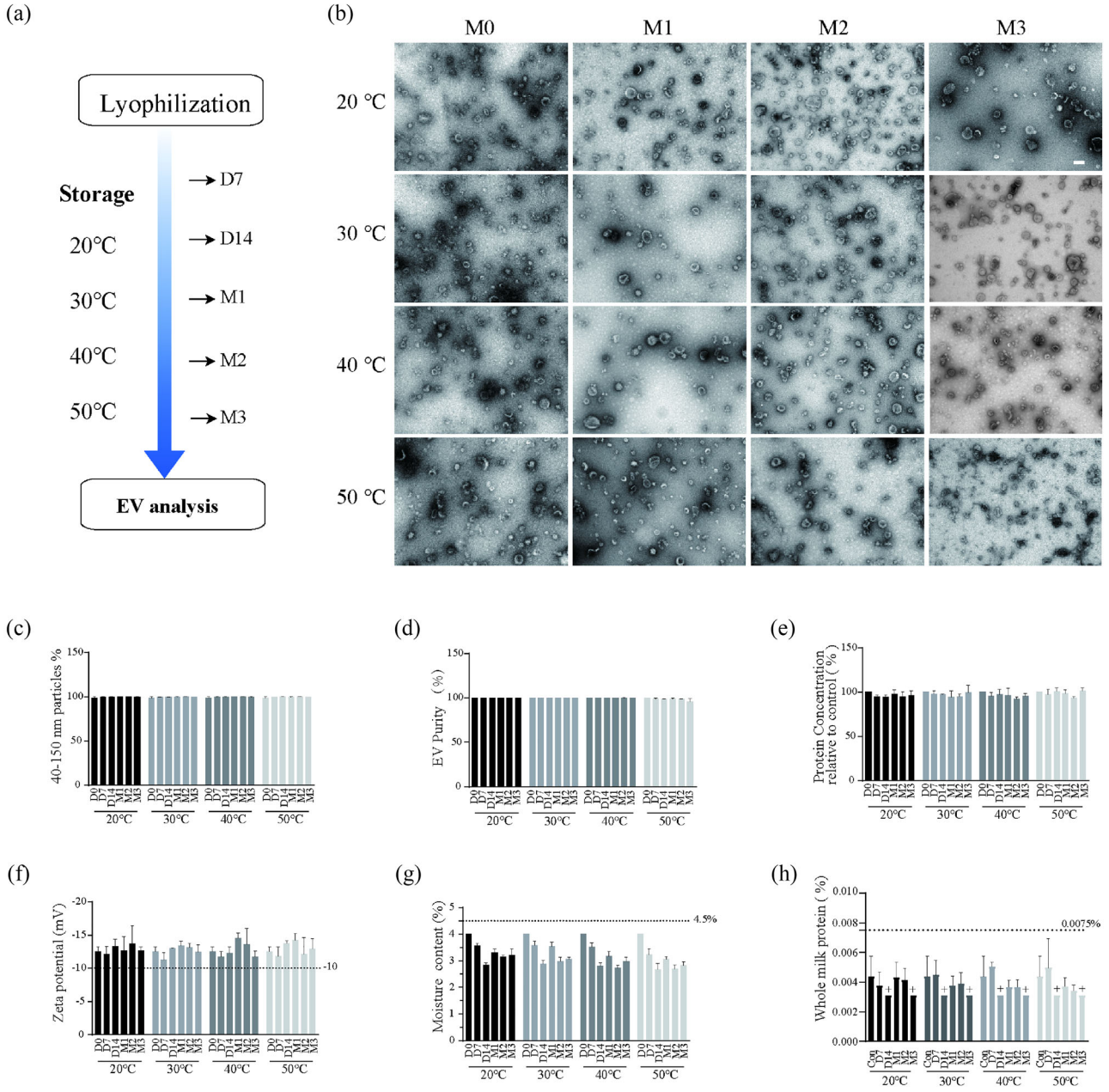



**FIGURE 4** Stability assessment of lyophilized bovine milk-derived exosomes (MK-Exo) under short-term storage conditions. (a) MK-Exo was lyophilized, and aliquots were stored at 20°C, 30°C, 40°C or 50°C and then analysed at different time points. "D" represents "Day" and "M" represents "Month." (b–h) Analysis of morphology, particle size, purity, protein concentration, zeta potential, moisture content and whole-milk protein content of lyophilized MK-Exo at various temperatures and time points. Scale bar = 500 nm. Error bars represent the standard deviation of three repetitive experiments. "+" indicates not detected.

**TABLE 3** $R^2$ and Pearson's R values of linear correlation curves for M1, M2 and M3.

| | M1 | | M2 | | M3 | |
|---|---|---|---|---|---|---|
| T (°C) | $R^2$ | Pearson's R | $R^2$ | Pearson's R | $R^2$ | Pearson's R |
| 20 | 0.86589 | 0.93062 | 0.89095 | 0.94397 | 0.90619 | 0.952 |
| 30 | 0.88053 | 0.93844 | 0.89646 | 0.94688 | 0.90259 | 0.95011 |
| 40 | 0.88427 | 0.94043 | 0.87388 | 0.93464 | 0.78787 | 0.88782 |
| 50 | 0.89878 | 0.94811 | 0.86938 | 0.9325 | 0.90425 | 0.95099 |

### 3.7 | Stability studies of the lyophilized MK-Exo under long-term storage condition

Furthermore, we assessed the stability of MK-Exo at specific time points over a period of 24 months at a storage temperature (2°C–8°C) easily achievable for transport (Figure 5a). Notably, there were no significant alterations in the morphological features, particle size, purity, protein concentration (standard line: > 90%), moisture content, and whole-milk protein content of MK-Exo at any evaluation point during the 24 months storage period (Figure 5b–e, 5g,h and Figures S8–S9). Additionally, the Zeta potential remained within the set standard, defined as an absolute value of >10 mV (Figure 5f).

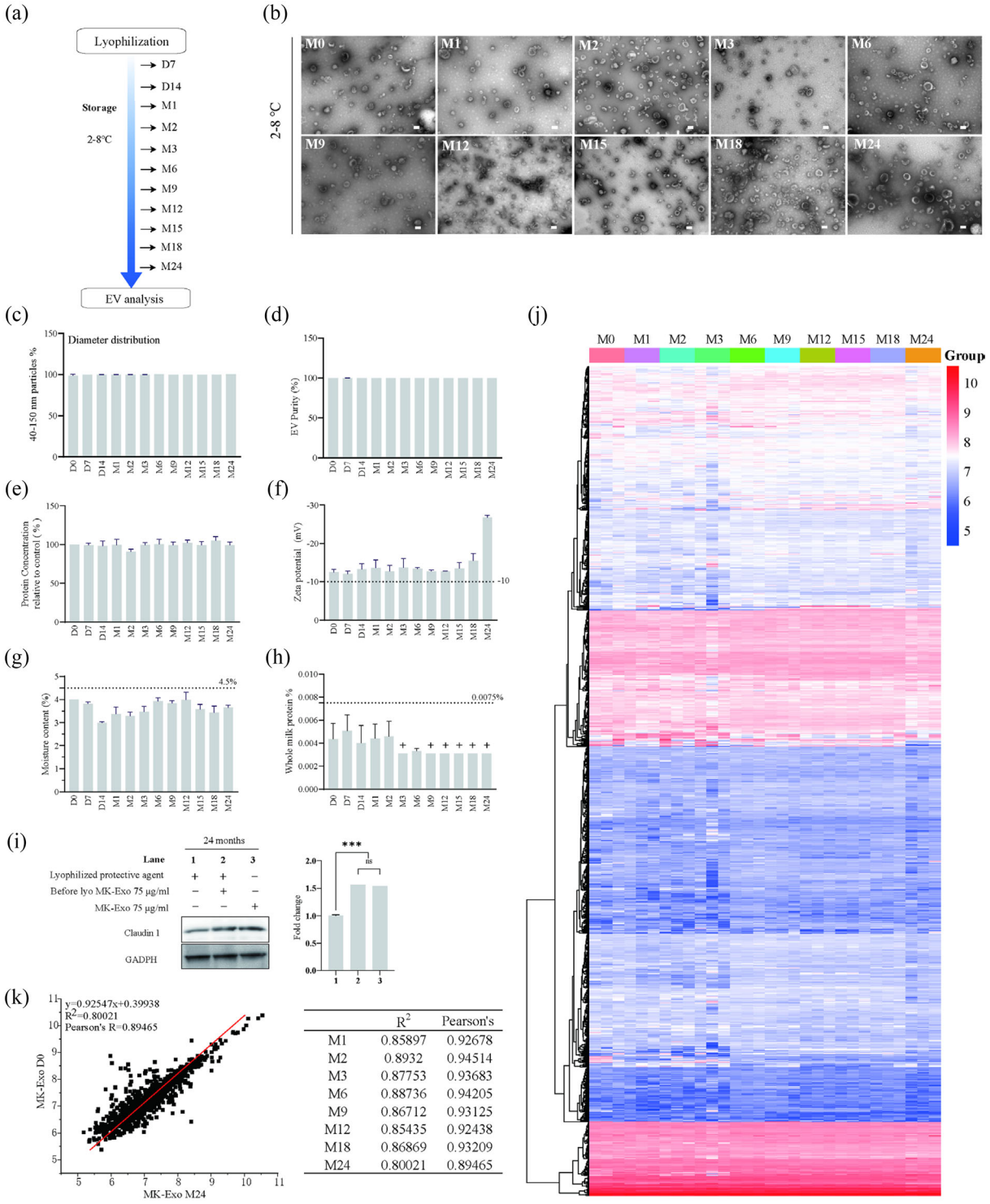
Western blotting showed that MK-Exo activity was not affected by lyophilization and storage duration, as evidenced by upregulated claudin-1 protein expression in Caco-2 cells following treatment with lyophilized MK-Exo stored for 15 and 24 months. Overall, these results indicate that the biological activity of lyophilized MK-Exo remained intact even after 24 months of storage at 2°C–8°C (Figure 5i and Figure S10). Additionally, typical MK-Exo marker proteins were detected at all evaluation points (Table S19). Importantly, the results of the Western blot analysis are shown in Figure S11. Moreover, heatmap analysis revealed no significant differences in proteome among the groups (Figure 5j), proteome data is provided in the supporting file. Furthermore, linear correlation analysis of the proteome data showed a high correlation between D0 and M24, indicating minimal proteomic alterations across different time points (Figure 5k and Figure S12). Collectively, these results indicate that the physicochemical properties and biological activity of lyophilized MK-Exo remain largely unchanged after storage at 2°C–8°C for a period of 24 months. Based on the result of stability experiments, the shelf life of MK-Exo at 4°C was predicted to be 1455.09 days. However, these predictions may not account for factors such as significant temperature differences and transport conditions.

## 4 | DISCUSSION

In the present study, we developed a novel lyophilization method for MK-Exo. Stability experiments revealed that the lyophilized MK-Exo can be stored for an extended period without significant degradation. Notably, lyophilized MK-Exo remained stable for up to 3 and 2 months under short-term storage conditions of 40°C and 50°C, respectively, indicating its suitability for conventional long-distance transportation. Additionally, lyophilized MK-Exo remained stable for at least 24 months under long-term storage condition of 4°C.

Liquid MK-Exo has been confirmed to endure storage at -80°C, although not for extended durations. Munagala et al. reported that the size of liquid MK-Exo did not change significantly after storage at -80°C for 18 months, consistent with the particle size results obtained in this study. In the present study, there was a significant decline in the purity of MK-Exo after 8 months of storage, which was attributed to proteins and nucleic acids secreted by fragmented exosomes. However, there were no significant changes in the morphology or particle size of MK-Exo, underscoring the importance of purity in MK-Exo. Typically, laboratory storage for exosomes involves freezing at -80°C (Agrawal et al., 2017; Lorincz et al., 2014; Munagala et al., 2016). However, some reports suggest that exosomes may exhibit instability during prolonged storage at -80°C. For instance, Maroto et al. (2017) demonstrated that airway exosomes stored at -80°C for 4 days showed morphological changes. Similarly, Wu et al. (2015) suggested that direct freezing could impact the stability of SKBR3 cell exosome membranes and degrade the samples. Overall, these inconsistencies in results following storage at -80°C may be attributed to variations in exosome sources, qualities and preparation processes. Exosomes derived from different cells or biological samples may have varying lipid, RNA, and protein compositions (Katsuda et al., 2013), leading to differences in membrane stability. Moreover, the stability of various RNA and protein components at -80°C may vary. Our laboratory has observed discrepancies in the proteomic profiles, physicochemical properties, and biological activities of exosomes obtained from different sources (data not shown). Furthermore, disparate cell culture techniques and purification processes can lead to harvested exosome samples containing varying levels and compositions of impurities. Overall, these factors may have contributed to the variations observed in exosome stability during storage at -80°C.

Notably, there is no universally applicable lyophilization method for different EVs. For example, the application of the lyophilization described by Geeurickx et al. (2019) resulted in a cake that was atrophied, collapsed and viscous, with a high moisture content and reduced Zeta potential. In contrast, the application of this method to freeze-dry MSC- and Expi293F cell-derived exosomes resulted in decreased exosome purity, although the appearance and characteristics (data not shown) were satisfactory. The unsatisfactory outcome could be attributed to the incompatibility of the lyophilization method for MK-Exo. Owing to the heterogeneity among exosomes sourced from diverse origins or prepared through varying processes, a tailored lyophilization technique is necessary. Refined isolation methods have revealed heterogeneity in exosomes, including differences in size, content, functional impact on recipient cells, and cellular origins (Zhang et al., 2018). Distinct exosome subsets, characterized by specific size ranges, have been identified, each possessing varying protein or RNA compositions. These variations may affect the lyophilization process or formulation, leading to lyophilization failure. Therefore, obtaining stable, high-quality exosomes and developing suitable lyophilization methods present significant challenges, highlighting the need for further research and optimization. Establishing standardized lyophilization methods for different types of EVs is crucial to preserve their integrity, functionality, and characteristics throughout the lyophilization process.




| | $R^2$ | Pearson's |
|---|---|---|
| M1 | 0.85897 | 0.92678 |
| M2 | 0.8932 | 0.94514 |
| M3 | 0.87753 | 0.93683 |
| M6 | 0.88736 | 0.94205 |
| M9 | 0.86712 | 0.93125 |
| M12 | 0.85435 | 0.92438 |
| M18 | 0.86869 | 0.93209 |
| M24 | 0.80021 | 0.89465 |

**FIGURE 5** Stability of lyophilized bovine milk-derived exosomes (MK-Exo) under long-term storage at 2°C–8°C. (a) Lyophilized MK-Exo aliquots were stored at 2°C–8°C and analysed at different time points. (b–h) Analysis of the morphology, particle size, purity, protein concentration, zeta potential, moisture content, and whole-milk protein content of lyophilized MK-Exo during long-term storage. (i) Western blot analysis of claudin-1 and GAPDH expression, with grayscale-based band analysis. (j) Proteome heatmap showing the profile of lyophilized MK-Exo after 24 months of storage at 2°C–8°C. (k) Linear correlation analysis of the proteome between D0 and M24, with accompanying tables showing $R^2$ and Pearson's R values for linear analysis between other time points and D0.

In the present study, we calculated the validity storage period following the procedure described in document *YY/T 0681.1-2018 Test Methods for Sterile Medical Device Packaging*. Notably, the theoretically calculations indicated that MK-Exo can be stored for up to 4 years under the long-term condition of 2°C–8°C, exceeding the duration of our current stability verification time. Overall, the theoretical data indicate the need to extending the storage time in future stability studies.

At the 24th month under the storage conditions of 2°C–8°C, the zeta potential of MK-Exo exhibited a significantly higher absolute value than at the previous time points, suggesting improved stability over time. The enhanced stability of the lyophilized exosomes can be attributed to their dry environment, which minimizes their interactions with the environment and reduces potential reaction and aggregation. Overall, the stability of the lyophilized EVs is expected to increase gradually over time, although further experimental evidence is required to validate this hypothesis.

MK-Exo are rich in both proteins and lipids. Major milk fat globule membrane (MFGM) proteins have been reported as the most abundant proteins in MK-Exo (Reinhardt et al., 2012). MFGM proteins play an important protective role against bacteria, viruses and protozoa (Cavaletto et al., 2004). Fuller et al. (2013) showed that whole buttermilk and cheese whey MFGM inhibited rotavirus in a dose-dependent manner. Additionally, Skotland et al. (2017) reported elevated levels of phosphatidylcholine, phosphatidylethanolamine, cholesterol, and phos-phatidylserine in MK-Exo, suggesting the potential applications of MK-Exo in the food industry to enhance the nutritional value of food products. For instance, the addition of MK-Exo to dairy products can augment both protein content and absorption rates, thereby increasing the overall nutritional value of the product. MK-Exo contains various bioactive molecules, such as antimicrobial peptides, immunomodulators (Wang et al., 2023), and antioxidants (based on MK-Exo proteomics data not shown), which impart unique functional properties to food items. Additionally, incorporating MK-Exo into beverages or foods can enhance the antioxidant capacity, delay oxidative deterioration, improve texture and enhance stability.

MK-Exo shows promising potential in skincare products. Research indicates that they can improve skin elasticity, reduce wrinkles, and exert skin repair and anti-ageing effects (Han et al., 2022). Therefore, incorporating MK-Exo into cosmetics and skincare products can enhance their moisturizing, anti-wrinkle and whitening effects (Lu et al., 2023). MK-Exo can also serve as an effective carrier in orally administered drug delivery systems. Their excellent stability and biocompatibility enable them to protect drugs from degradation by acidic gastric fluids and intestinal enzymes, providing a novel approach to oral drug delivery (Lin et al., 2020).

Considering that MK-Exo are derived from milk, there may be some ethical issues involved. For example, animal welfare, religious prohibitions, and some strict vegan boycotts of dairy products. Therefore, the use of well-sourced, protein-rich, and inexpensive beans as a starting material for exosome production is promising.

Conclusively, the development of freeze-drying methods for MK-Exo and the assessment of their long-term stability holds great significance. These efforts not only enhance the preservation stability of milk-derived exosomes, but also promote further research and development, opening up diverse possibilities for future applications and commercialization.

## AUTHOR CONTRIBUTIONS

**Lu Lu**: Conceptualization (lead); resources (lead) and supervision (lead). **Chunle Han**: Conceptualization (equal); data curation (lead); methodology (lead) and validation (lead). **Miao Wang**: Methodology (lead); supervision (lead) and validation (lead). **Huanqing Du**: Project administration (equal). **Ning Chen**: Visualization (equal); writing—original draft (equal) and writing—review and editing (equal). **Mengya Gao**: Visualization (equal); writing—original draft (equal) and writing—review and editing (equal). **Na Wang**: Methodology (equal) and validation (equal). **Dongli Qi**: Methodology (equal) and validation (equal). **Wei Bai**: Project administration (equal). **Jianxin Yin**: Data curation (equal); formal analysis (equal) and investigation (equal). **Fengwei Dong**: Funding acquisition (equal). **Tianshi Li**: Funding acquisition (equal); project administration (equal) and supervision (equal). **Xiaohu Ge**: Funding acquisition (lead); project administration (lead) and supervision (lead).

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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