# Advancements in Lyophilized Exosomes for Enhanced Stability and Therapeutic Applications

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## Introduction

Extracellular Vesicles (EVs), particularly exosomes, are nanoscale vesicles secreted by cells that play critical roles in intercellular communication. They have emerged as promising candidates for diagnostics and therapeutics in a wide range of diseases, including cancer, neurodegenerative disorders, and inflammatory conditions. Despite their potential, the clinical translation of EV-based therapies is hindered by the need for ultra-low temperature storage (−80°C), which complicates logistics and increases costs.

Lyophilization, or freeze-drying, presents a viable solution by enabling the development of stable, room-temperature formulations. This method removes water from EV preparations under low pressure and temperature, preserving their structural and functional integrity. By eliminating the need for cold-chain logistics, lyophilization could significantly enhance the scalability, accessibility, and global distribution of EV-based products.

However, the success of this approach depends on the careful selection of buffer systems and cryoprotectants that can protect EVs during the drying and rehydration processes. Cryoprotectants such as sugars, polymers, and amino acids can stabilize the lipid bilayer and protein components of EVs, preventing aggregation or degradation. Understanding the interplay between these components is essential for developing a robust lyophilized EV platform suitable for clinical and commercial applications.

## Methods

To develop a robust lyophilized EV platform, we conducted a multi-phase optimization study:

1. **Buffer Screening**:
   Freeze-thaw (FT) studies were performed using five different EV buffers to identify the most protective formulation. Five buffers includes Phosphate Buffered Saline (PBS), Sodium phosphate, Potassium phosphate, HEPES, and HEPES Buffered Saline (HBS). The optimized buffer was selected based on its ability to maintain particle concentration and size over three FT cycles.

2. **Cryoprotectant Evaluation**:
   Eight cryoprotectant (CPA) combinations—comprising both single agents and blends—were tested for their ability to preserve EV particle size and concentration post-lyophilization. Nanoparticle Tracking Analysis (NTA) was used for quantitative assessment.

3. **Functional Assays**:

   a) **Cell Migration Assay**: Conducted using primary human dermal fibroblasts (ATCC®PCS-201-012™) and keratinocytes (ATCC® CRL-4048™ [hTERT immortalized]) to assess pro-migratory effects.

   b) **LPS Challenge Assay**: Performed using ThawReady™ THP-1 NF-κB-Luc2 monocytes (ATCC® TIB-202-NFkB-LUC2-AR™) to evaluate anti-inflammatory activity.

## Buffer Screening

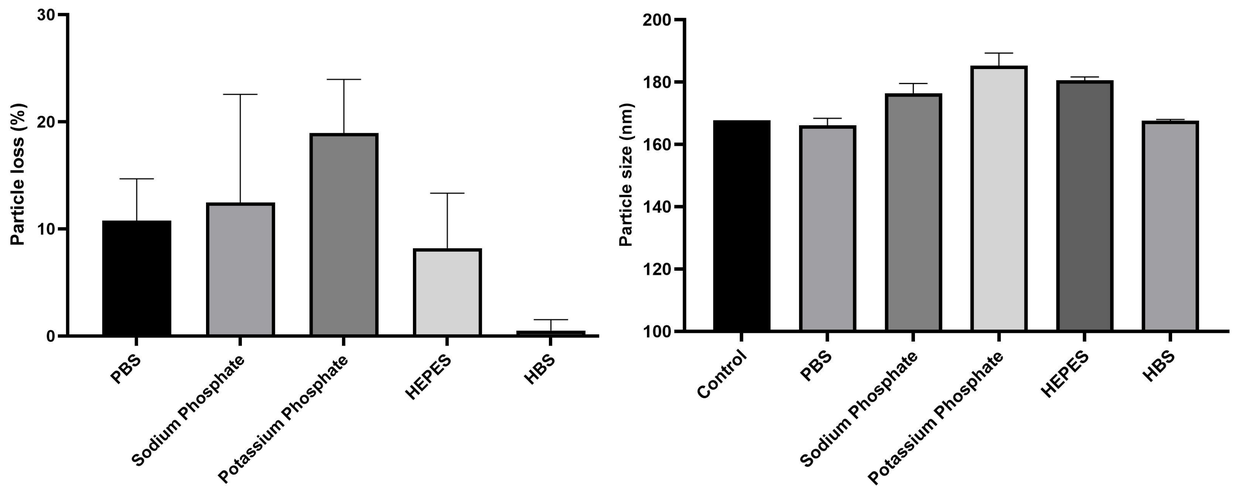



**Figure 1: Comparison of EV particle size and concentration across five buffer systems after three freeze-thaw cycles.** HBS demonstrated the highest preservation of EV characteristics.

To identify the most suitable buffer for preserving EV integrity during freeze-thaw (FT) cycles, five buffer systems were systematically evaluated: Phosphate Buffered Saline (PBS), Sodium Phosphate, Potassium Phosphate, HEPES, and HEPES Buffered Saline (HBS). These buffers were selected based on their common use in biological formulations and their potential to stabilize nanoparticles.

Among the tested formulations, HBS and PBS demonstrated superior performance in maintaining EV particle concentration and size across three FT cycles. Notably, HBS consistently preserved EV integrity with minimal particle loss or aggregation, indicating its enhanced protective capacity during thermal stress. This superior performance is likely due to the buffering capacity and ionic composition of HBS, which may better stabilize the lipid bilayer and protein components of EVs.

Given its ability to maintain both structural and quantitative EV parameters, HBS was selected as the optimal buffer for downstream lyophilization studies. Its consistent performance across replicates underscores its potential as a standard formulation for EV preservation in both research and therapeutic contexts.

## Cryoprotectant Evaluation

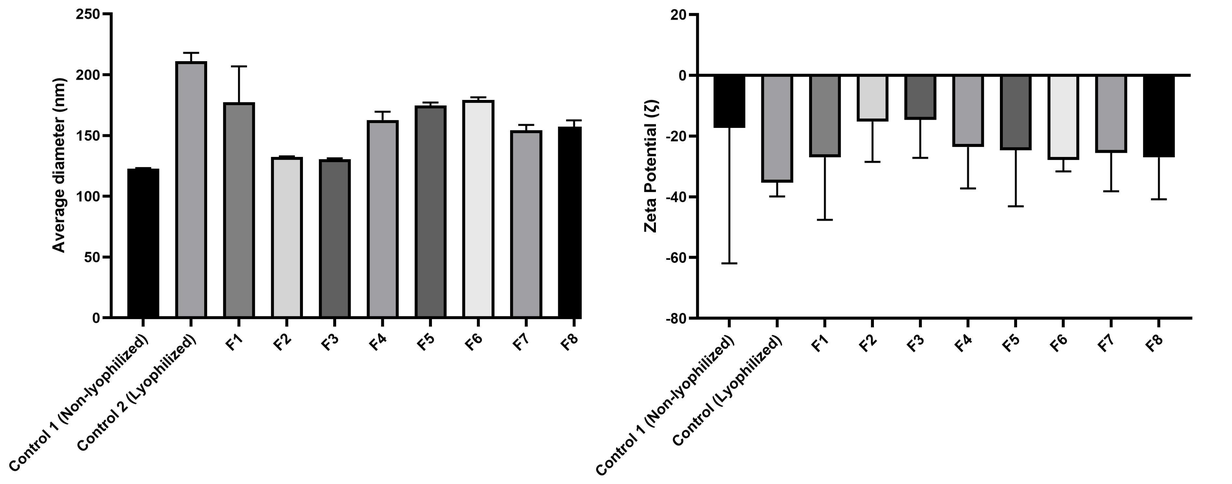



**Figure 2: Average particle diameter and zeta potential of EVs post-lyophilization across different cryoprotectant formulations.** The experiments were performed in triplicate (n=3). F2 and F3 maintained optimal characteristics.

Eight different cryoprotectant (CPA) formulations were tested to determine their ability to preserve EV characteristics post-lyophilization. Among all, **F2** and **F3** demonstrated the most favorable outcomes in preserving the average particle diameter of lyophilized EVs, indicating minimal aggregation or structural disruption during the lyophilization process. In contrast, Control 2, which consisted of EVs lyophilized without any CPA, exhibited a significantly increased particle size ($p<0.01$), suggesting substantial vesicle fusion or aggregation.

Zeta potential is a key indicator of colloidal stability. In this study, **F2 and F3** formulations again outperformed others by maintaining zeta potential values within the ideal range—neither too positive nor too negative—thus supporting the electrostatic stability of the lyophilized EVs.

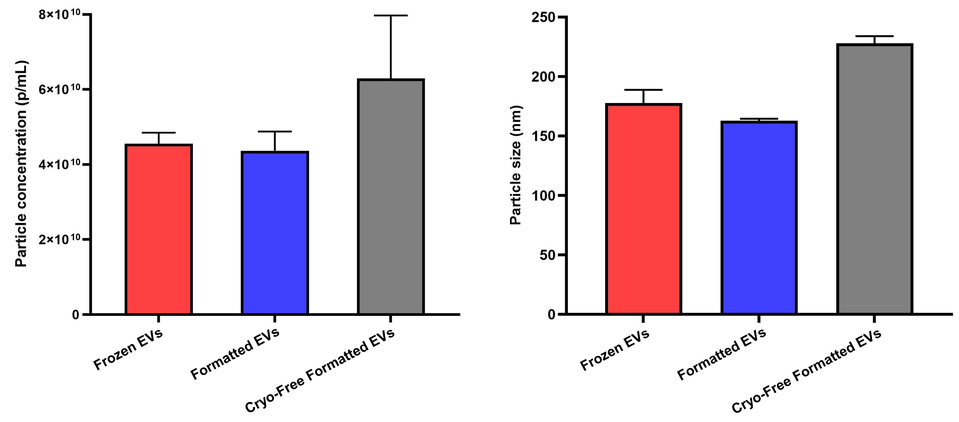
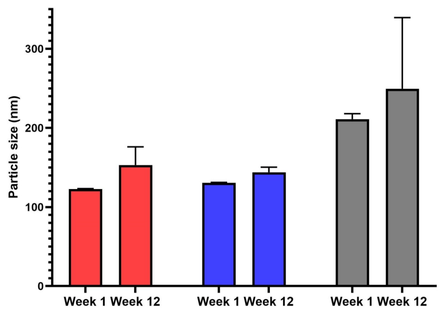



**Figure 3: Average particle diameter and particle concentrations of EVs post-lyophilization.** Frozen EVs, Formatted EVs (F3) and cryo-free formatted EVs were compared.

F3-formatted EVs demonstrated a remarkable retention of nanoscale size and particle count, closely mirroring the characteristics of the non-lyophilized control. In contrast, cryo-free EVs exhibited significant deviations in both parameters, indicative of vesicle aggregation and structural compromise.




Formatted EVs demonstrated excellent stability, with no significant changes ($p<0.08$) in particle size compared to cryo-free formatted EVs upon a 12-week stability study indicating that the formatted EVs retained their structural integrity and did not undergo aggregation or degradation under standard storage conditions.

**Figure 4: Stability analysis of Frozen EVs, Formatted EVs (F3), and cryo-free formatted EVs were performed for 12 weeks.**

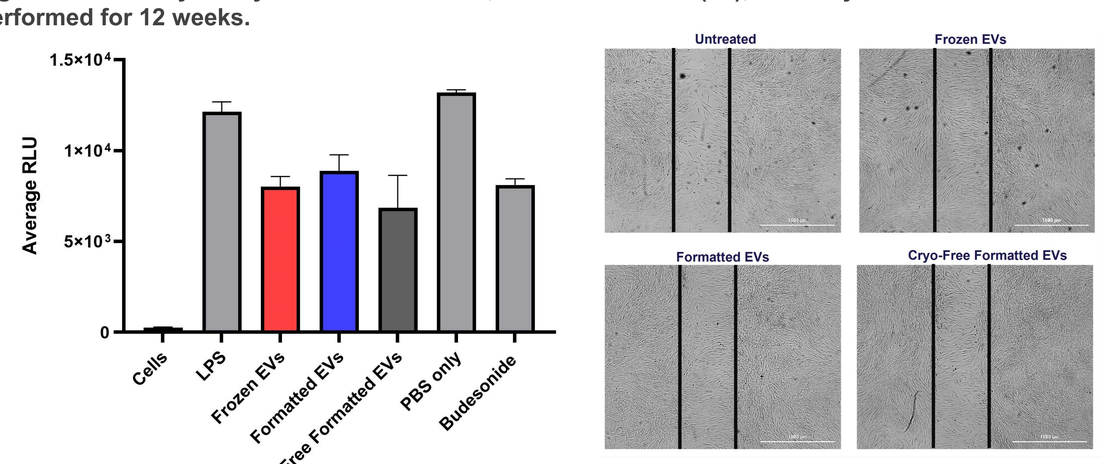



**Figure 5: Functional analysis of lyophilized EVs in LPS-induced inflammation model using ThawReady™ THP-1 NF-κB-Luc2 monocytes and a cell migration assay.**

F3-formatted EVs significantly reduced LPS-induced inflammation in THP-1 NF-κB-Luc2 monocytes exhibiting a response comparable to that of frozen EVs, indicating that the lyophilization process did not impair the immunomodulatory functions of EVs. Formatted EVs exhibited comparable wound healing performance as that of frozen EVs in the cell migration assay

## Conclusion

Lyophilized EVs formulated with optimized buffers and cryoprotectants demonstrated excellent structural stability and preserved biological activity. This approach enables the development of room-temperature-stable EV products, facilitating broader clinical and research applications. The platform offers a scalable solution to overcome current storage and distribution challenges in EV-based therapeutics.

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