Development and Characterization of Lyophilized Extracellular Vesicles (EVs) for Enhanced Stability and Cellular Repair

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Introduction

Extracellular vesicles (EVs), particularly exosomes, are nanoscale vesicles that are secreted by all cell types and play critical roles in intercellular communication. They have emerged as promising candidates for both diagnostic and therapeutic applications in a wide range of diseases, including cancer, neurodegenerative disorders, and inflammatory conditions. Despite their broad research and translational potential, widespread implementation of EV-based products is hindered by the need for ultra-low temperature storage (−80°C), which complicates logistics, limits accessibility, and increases operational 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 the biophysical and functional properties of 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

1. **EV Production:** EVs were produced from hTERT-immortalized human mesenchymal stromal cells (MSCs; ATCC® SCRC-4000™) cultured in a fixed-bed bioreactor and isolated from conditioned medium prior to formulation and lyophilization.

Explore EVs

2. **Buffers Screening**: Freeze-thaw (FT) studies were performed using five different EV buffers to identify the most protective formulation: 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.

3. **Cryoprotectants 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.

4. **Characterization Assays:**

   A. **EV Surface Marker Screening:** EV-associated surface proteins (CD63, CD81, CD9) were assayed using ultrasensitive electrochemiluminescence (ECL) immunoassays with EV calibrator standards (Meso Scale Diagnostics).

   B. **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.

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

Buffer Screening

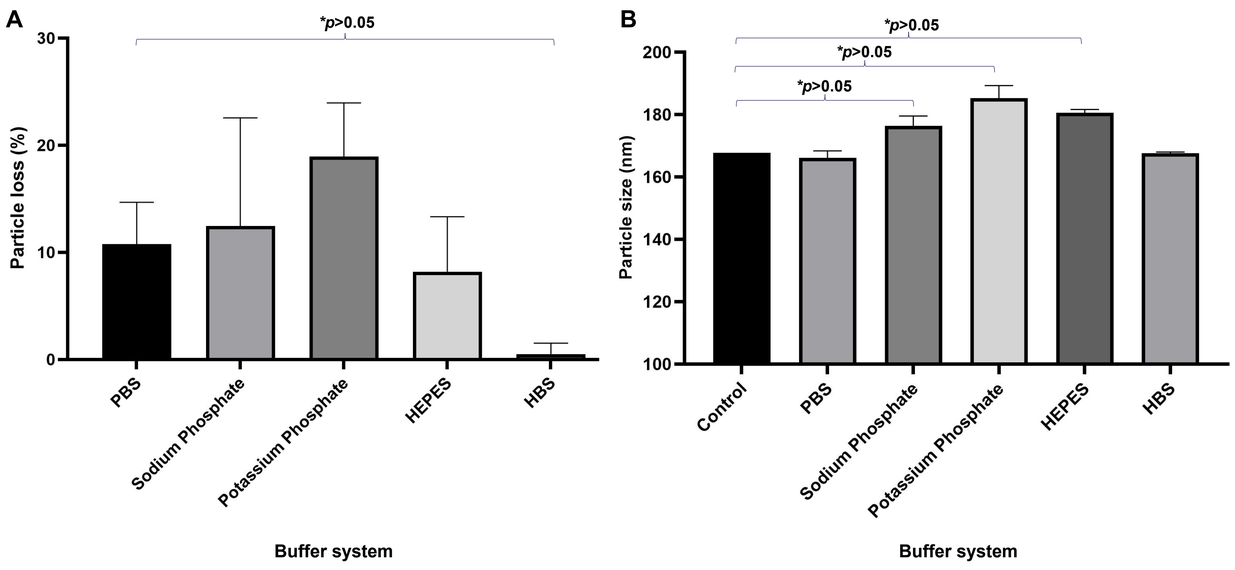

Figure 1: Comparison of EV characteristics across five buffer systems. (A) Percentage particle loss and (B) EV particle size was measured following three freeze-thaw cycles across five buffer systems. HBS demonstrated the greatest preservation of EV characteristics across both parameters.

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

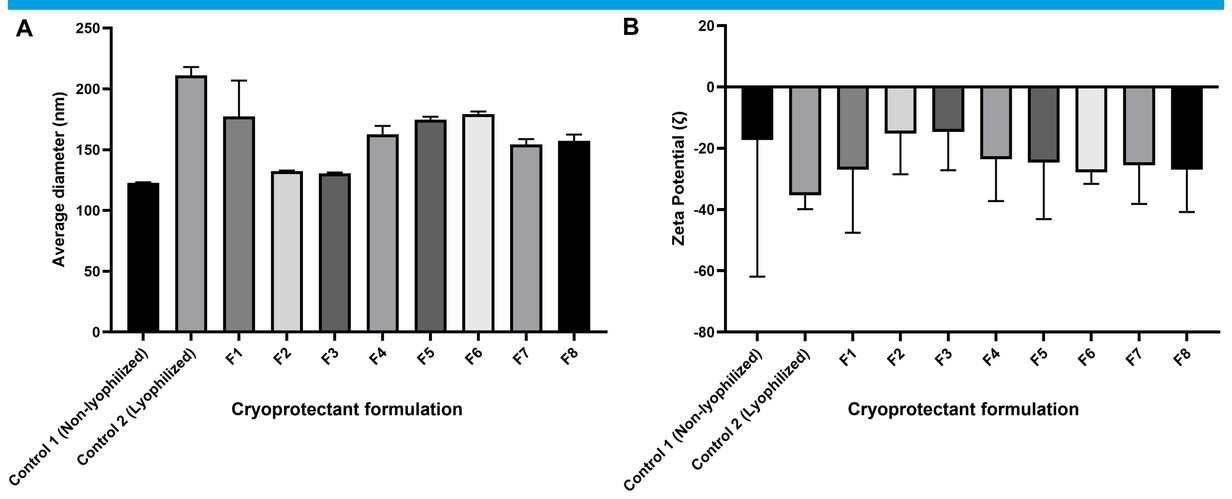

Figure 2: Effect of cryoprotectant formulation on EV characteristics following lyophilization. (A) Average EV particle diameter and (B) EV zeta potential measured after lyophilization in eight cryoprotectant (CPA) formulations (F1-F8). Experiments were performed in triplicate (n=3). F2 and F3 maintained 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.

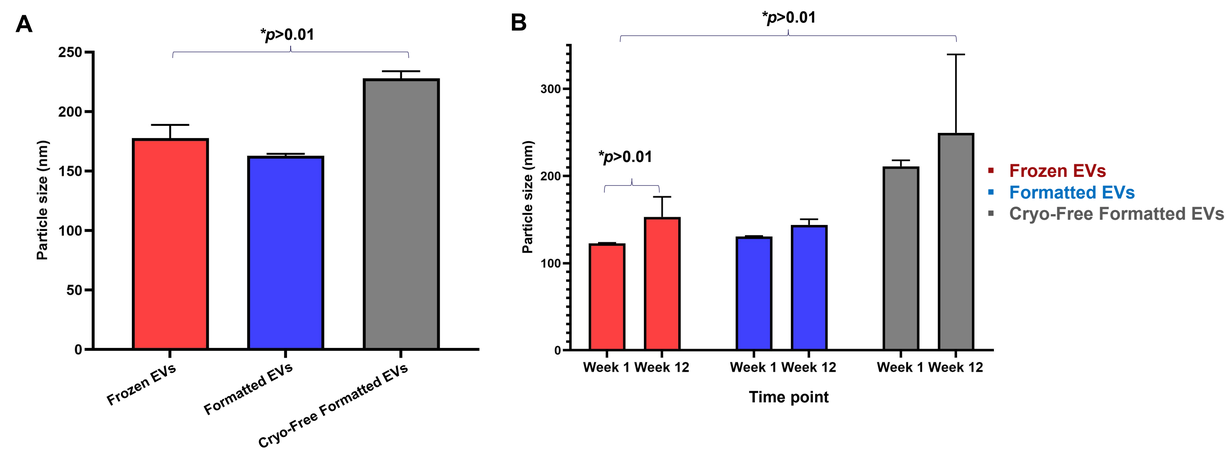

Figure 3: Comparison of EV particle size and stability following lyophilization. (A) Average particle size of frozen EVs, formatted EVs (F3) and cryo-free formatted EVs. (B) Stability analysis of frozen EVs, formatted EVs (F3), and cryo-free formatted EVs were performed for 12 weeks. Formatted EVs: Lyophilized EVs in final format.

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.

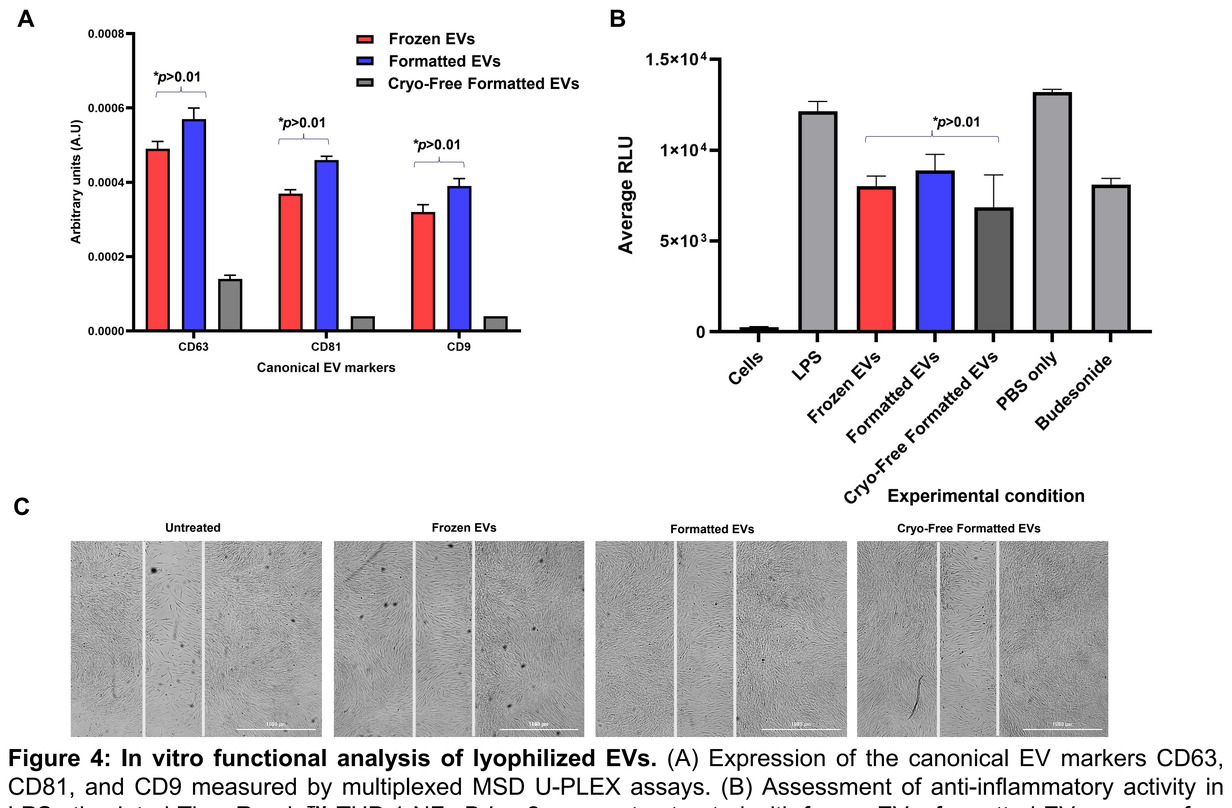

Figure 4: In vitro functional analysis of lyophilized EVs. (A) Expression of the canonical EV markers CD63, CD81, and CD9 measured by multiplexed MSD U-PLEX assays. (B) Assessment of anti-inflammatory activity in LPS-stimulated ThawReady™ THP-1 NF-κB-Luc2 monocytes treated with frozen EVs, formatted EVs, or cryo-free formatted EVs., (C) Cell migration assay demonstrating retention of EV biological activity following lyophilization and formulation.

F3-formatted EVs significantly reduced LPS-induced inflammation in THP-1 NF-κB-Luc2 monocytes exhibiting a response comparable to that of frozen EVs. Formatted EVs consistently yielded the highest recovery and detection signals for all three classical EV markers (CD63, CD81, and CD9). 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 preserved EV particle size, recovery, surface marker expression, and functional activity after reconstitution. Formatted EVs maintained CD63, CD81, and CD9 detectability, reduced LPS-induced inflammatory signaling, and supported cell migration activity comparable to frozen EVs. The platform offers a scalable solution to overcome current storage and distribution challenges in EV-based therapeutics.

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