Comparative study on the impact of storage conditions on the physicochemical properties of red-blood cell derived extracellular particles

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BACKGROUND AND AIM OF THE PROJECT

Recently, drug delivery systems based on natural sources have gained significant interest into the scientific community. Among them, erythrocyte-derived particles have emerged as a promising class of bioinspired nanocarriers, combining intrinsic biocompatibility, bioavailability, low immunogenicity and prolonged circulation time with the potential for surface functionalization and cargo encapsulation. Erythrocyte-derived extracellular particles (EPs) can be exploited as nanocarriers either in their native form or following haemoglobin depletion through a hypotonic treatment, generating the so-called ghost particles. Here, we investigated how the ghosting process affects the yield and physicochemical quality of artificially generated RBC-derived EPs (here referred to as Nanoerythrosomes and NanoEs). We evaluated the stability of these nanocarriers during frozen storage, a critical requirement for their practical implementation in drug delivery applications, since freeze–thaw conditions may compromise vesicle integrity, cargo retention, and overall delivery performance. In this context, the use of cryoprotective formulations was explored to improve vesicle preservation and minimize storage-induced structural alterations. Native and ghost-NanoEs were comparatively analysed immediately after isolation and after 21 and 60 days of storage at −80 °C in PBS, 4% trehalose, or a commercial storage buffer.

EXPERIMENTAL PLAN

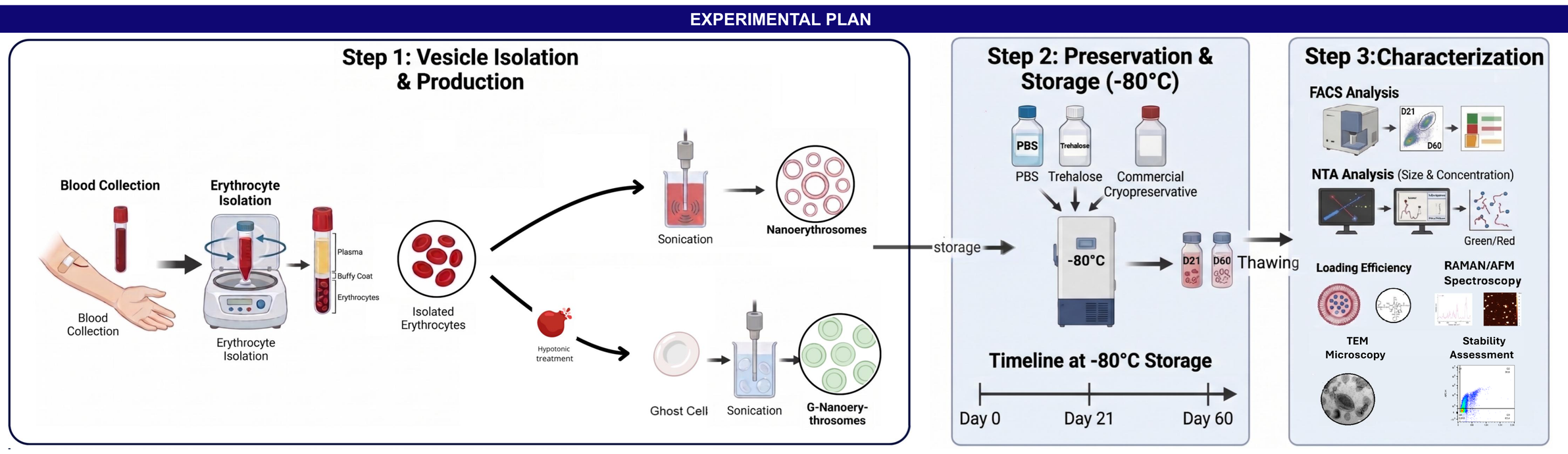

Figure 1. Experimental plan for NanoEs and G-NanoEs production, storage and characterization. Particles were produced starting using blood from healthy donors. Erythrocytes were isolated from whole blood by sequential centrifugation and using leukodepletion filters. Ghost-cells were obtained through an hypotonic treatment .NanoEs and G-NanoEs were produced using a sonication method then stored using formulations, PBS, 4% trehalose and JotBody® storage buffer, at -80°C for 21 and 60 days. The two populations of particles were characterized by NTA, Zeta-Potential and FACS to assess post-thaw recovery, size distribution, colloidal stability and biomarker preservation. Moreover the Particles were loaded with a model fluorescent drug (Oregon-green Paclitaxel) to compare their loading efficiency after storage.

FRESH NanoES AND G-NanoES CHARACTERIZATION

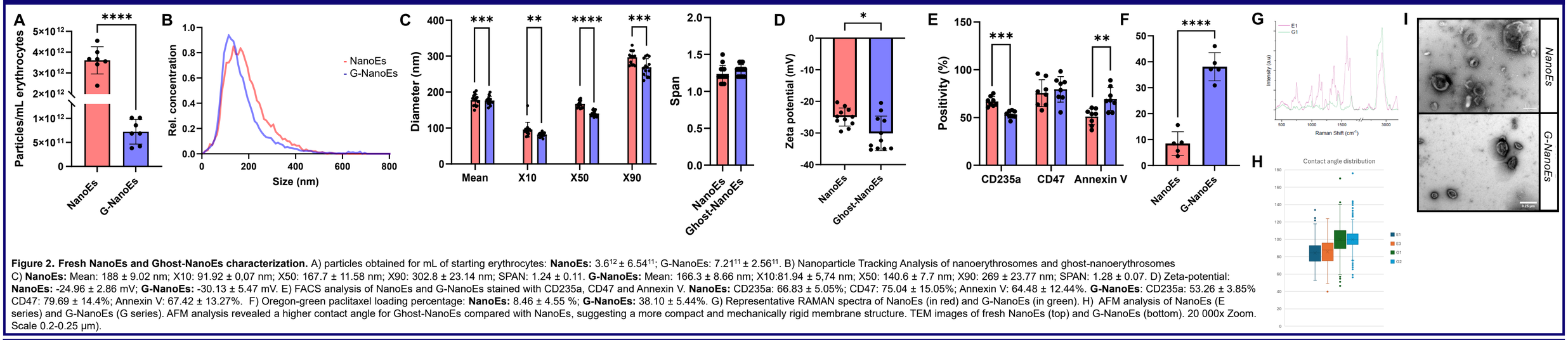

Figure 2. Fresh NanoEs and Ghost-NanoEs characterization. A) particles obtained for mL of starting erythrocytes: **NanoEs:** $3.6^{12} \pm 6.54^{11}$; G-NanoEs: $7.21^{11} \pm 2.56^{11}$. B) Nanoparticle Tracking Analysis of nanoerythrosomes and ghost-nanoerythrosomes C) **NanoEs:** Mean: 188 ± 9.02 nm; X10: 91.92 ± 0,07 nm; X50: 167.7 ± 11.58 nm; X90: 302.8 ± 23.14 nm; SPAN: 1.24 ± 0.11. **G-NanoEs:** Mean: 166.3 ± 8.66 nm; X10:81.94 ± 5,74 nm; X50: 140.6 ± 7.7 nm; X90: 269 ± 23.77 nm; SPAN: 1.28 ± 0.07. D) Zeta-potential: **NanoEs:** -24.96 ± 2.86 mV; **G-NanoEs:** -30.13 ± 5.47 mV. E) FACS analysis of NanoEs and G-NanoEs stained with CD235a, CD47 and Annexin V. **NanoEs:** CD235a: 66.83 ± 5.05%; CD47: 75.04 ± 15.05%; Annexin V: 64.48 ± 12.44%. **G-NanoEs**: CD235a: 53.26 ± 3.85% CD47: 79.69 ± 14.4%; Annexin V: 67.42 ± 13.27%. F) Oregon-green paclitaxel loading percentage: **NanoEs:** 8.46 ± 4.55 %; **G-NanoEs:** 38.10 ± 5.44%. G) Representative RAMAN spectra of NanoEs (in red) and G-NanoEs (in green). H) AFM analysis of NanoEs (E series) and G-NanoEs (G series). AFM analysis revealed a higher contact angle for Ghost-NanoEs compared with NanoEs, suggesting a more compact and mechanically rigid membrane structure. TEM images of fresh NanoEs (top) and G-NanoEs (bottom). 20 000x Zoom. Scale 0.2-0.25 µm).

IMPACT OF STORAGE ON THE PHYSIOCHEMICAL PROPERTIES OF NanoEs AND G-NanoES

NanoEs

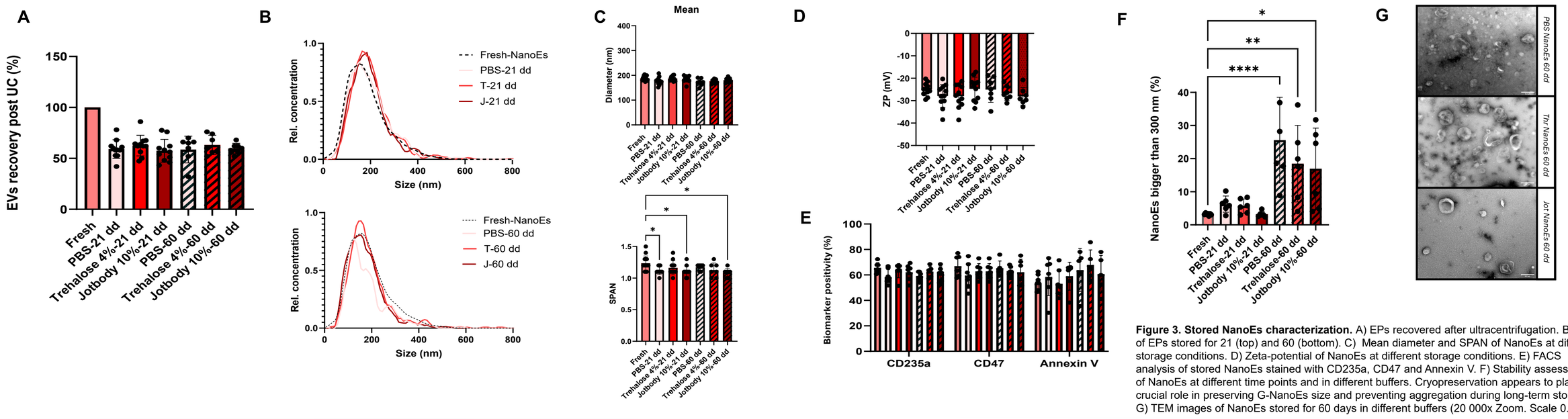

Figure 3. Stored NanoEs characterization. A) EPs recovered after ultracentrifugation. B) NTA of EPs stored for 21 (top) and 60 (bottom). C) Mean diameter and SPAN of NanoEs at different storage conditions. D) Zeta-potential of NanoEs at different storage conditions. E) FACS analysis of stored NanoEs stained with CD235a, CD47 and Annexin V. F) Stability assessment of NanoEs at different time points and in different buffers. Cryopreservation appears to play a crucial role in preserving G-NanoEs size and preventing aggregation during long-term storage. G) TEM images of NanoEs stored for 60 days in different buffers (20 000x Zoom. Scale 0.2 µm).

G-NanoEs

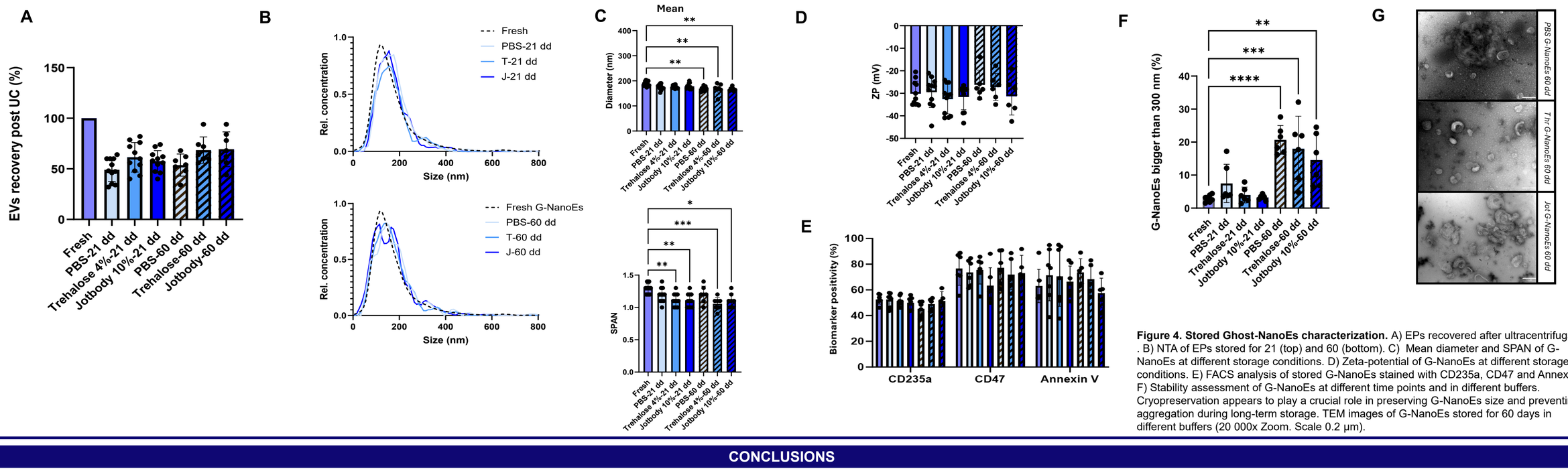

Figure 4. Stored Ghost-NanoEs characterization. A) EPs recovered after ultracentrifugation . B) NTA of EPs stored for 21 (top) and 60 (bottom). C) Mean diameter and SPAN of G-NanoEs at different storage conditions. D) Zeta-potential of G-NanoEs at different storage conditions. E) FACS analysis of stored G-NanoEs stained with CD235a, CD47 and Annexin V. F) Stability assessment of G-NanoEs at different time points and in different buffers. Cryopreservation appears to play a crucial role in preserving G-NanoEs size and preventing aggregation during long-term storage. TEM images of G-NanoEs stored for 60 days in different buffers (20 000x Zoom. Scale 0.2 µm).

CONCLUSIONS

G-NanoEs outperformed NanoEs, exhibiting more than a two-fold increase in Oregon Green-PTX uptake, highlighting the beneficial effect of haemoglobin depletion on loading capacity. No significant differences among formulations were observed in biomarker expression, size, or zeta potential. All cryopreservation conditions effectively reduced vesicle aggregation, highlighting their role in maintaining nanoparticle stability during short-term storage. However, after 60 days of cryopreservation, a marked increase in vesicle aggregation was observed across formulations, suggesting that prolonged storage negatively affects colloidal stability. Therefore, the use of freshly prepared nanoparticles remains preferable to ensure optimal particle stability.

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