Microfluidic-Engineered Immunoliposomes Targeting Cardiomyocytes: Optimization and In Vitro Evaluation for Fabry Disease Therapy

Luiz Henrique Mesquita Souza, Daniel Giuliano Cerri, Lívia Vieira Depieri, Maria Vitória Lopes Badra Bentley
University of São Paulo, School of Pharmaceutical Sciences of Ribeirão Preto, Brazil

lhmesquita.souza@usp.br
vbentley@usp.br

NanoGeneSkin Bentley's Laboratory

INTRODUCTION

Fabry disease is a lysosomal storage disorder caused by alpha-galactosidase A (GLA) deficiency, which leads to the pathological accumulation of globotriaosylceramide, primarily in the heart and kidneys. While conventional enzyme replacement therapy (ERT) with recombinant GLA is the standard of care, its long-term efficacy is limited by significant challenges, such as rapid clearance and poor tissue biodistribution (1,2). To address this, we synthesized polyethylene glycol (PEG)ylated immunoliposomes via a microfluidic method, achieving high encapsulation efficiency (EE%). These liposomes are engineered to specifically target cardiomyocytes, the primarily affected cell type, by recognizing integrin alpha-7 (ITGA7), a highly specific muscle tissue protein, facilitating active intracellular delivery (3).

METHODS

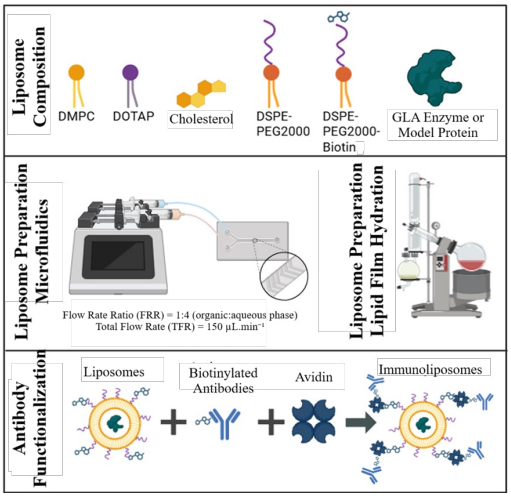

Figure 1: Methods utilized including liposome composition, preparation and antibody functionalization.

PEGylated liposomes were prepared via microfluidics (MF) at a 1:4 organic-to-aqueous flow rate ratio (FRR). Following overnight dialysis, residual ethanol was quantified by gas chromatography-mass spectrometry (GC-MS). Hydrodynamic size and polydispersity index (PDI) were assessed via dynamic light scattering (DLS), topography via tapping-mode atomic force microscopy (AFM) and Cryogenic Transmission Electron Microscopy (Cryo-TEM), and physicochemical stability was monitored over 60 days at 4 °C. Following the removal of free cargo via size exclusion chromatography (SEC), the encapsulation efficiency (EE%) of Alexa Fluor 647-conjugated bovine serum albumin (BSA) was measured fluorometrically. A Design of Experiments (DoE) optimized encapsulation based on phosphate-buffered saline (PBS) ionic strength, total flow rate (TFR), PEG, cationic lipid, and initial protein concentrations. Payload protection was confirmed via a 2.5 h trypsin challenge (37 °C), while antibody functionalization was verified via bicinchoninic acid (BCA) assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Internalization kinetics (1×10^9 NPs/mL) in differentiated H9c2 cardiomyocytes were quantified by flow cytometry at 15 min, 30 min, 4 h, and 24 h; to probe endocytic pathways, cellular uptake at the 15- and 30-min timepoints was evaluated both with and without a 1-h pre-incubation with 20 µg/mL chlorpromazine (CPZ). Immunofluorescence staining for ITGA7 and desmin was performed on differentiated H9c2 cardiomyocytes. Finally, nanoparticle-membrane interactions and intracellular localization within H9c2 cardiomyocytes were visualized using confocal microscopy after a 24-h incubation, applying 1×10^9 NPs/mL for live cells and 1×10^{10} NPs/mL for fixed cells.

RESULTS

Table 1: Physicochemical characterization of the optimized formulation following DoE methodology. Evaluated parameters are mean vesicle size, PDI, drug loading (DL%), EE%, and the absolute quantity of encapsulated protein per mL of formulation.

Size	PDI	DL%	EE%	Quantity of Protein Encapsulated per mL of Formulation
88.0 ± 8.9 nm	0.118 ± 0.019	2.88 ± 0.08 %	84.2 ± 2.3 %	14.8 ± 0.4 µg

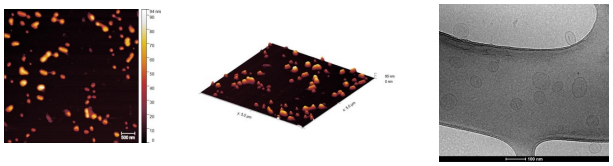

Figure 2: (Left) AFM imaging under dry conditions. **(Middle)** 3D AFM topographical reconstruction. **(Right)** Cryo-TEM imaging.

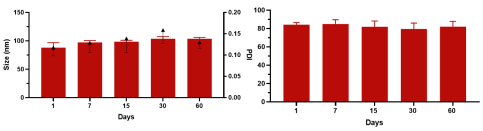

Figure 3: Stability assessment of formulation over a 60-day period at 4 °C **(Left)** Physical stability evaluated by monitoring the hydrodynamic size and PDI. **(Right)** Preservation of EE% over the same 60-day timeframe.

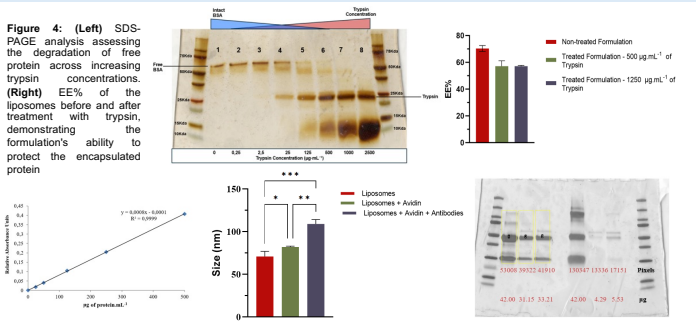

Figure 4: (Left) SDS-PAGE analysis assessing the degradation of free protein across increasing trypsin concentrations. **(Right)** EE% of the liposomes before and after treatment with trypsin, demonstrating the formulation's ability to protect the encapsulated protein

Figure 5: Determination of functionalization efficiency through the quantification of unconjugated antibody. **(Left)** Standard calibration curve generated for protein quantification using the BCA assay. **(Middle)** Stepwise increase in the hydrodynamic diameter following each stage of functionalization. **(Right)** SDS-PAGE followed by ImageJ densitometric analysis. After correcting with a non-biotinylated antibody control, the overall functionalization efficiency was calculated as 84.8 ± 2.6% based on ImageJ densitometry and 80.8 ± 5.9% based on the BCA assay.

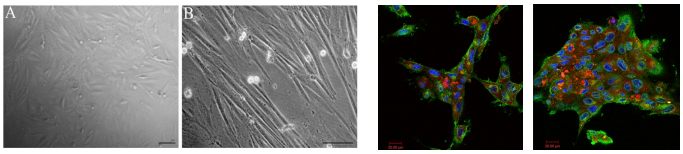

Figure 6. *In vitro* morphology, immunophenotyping, and cytotoxicity assessment. **(Left)** Representative microscopy images of H9c2 cells before differentiation (A, myoblasts) and after 10 days of differentiation (B, cardiomyocytes). **(Right)** Immunofluorescence staining of differentiated H9c2 cardiomyocytes for desmin (C) and ITGA7 (D). Nuclei are counterstained with DAPI (blue) and cell membranes with WGA (green).

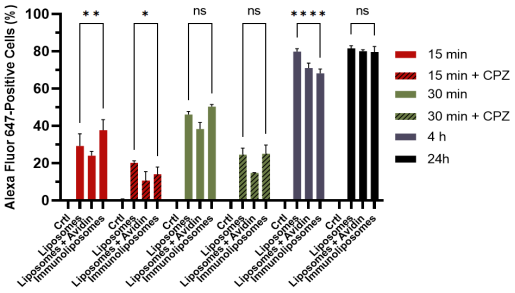

Figure 7. Internalization kinetics in differentiated H9c2 cardiomyocytes. Flow cytometry quantification of cellular uptake, expressed as the percentage of Alexa Fluor 647-positive cells. Early timepoints reveal a more pronounced uptake of immunoliposomes at 15 min compared to non-functionalized controls. Additionally, clathrin-mediated inhibition decreased immunoliposome internalization approximately 1.5-fold more than that of conventional liposomes. Extended timepoints show a delayed uptake peak at 4 h due to receptor saturation, followed by formulation equilibration at 24 h.

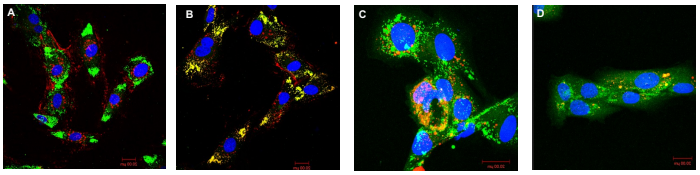

Figure 8. Intracellular distribution and membrane interaction of liposomal formulations in H9c2 cardiomyocytes. **(A, B)** Fixed cells incubated with (**A**) conventional liposomes and (**B**) anti-ITGA7 immunoliposomes (green). Cell membranes are stained with WGA (red) and nuclei with DAPI (blue). Pronounced membrane colocalization is exclusively observed for the immunoliposomes (**B**). **(C, D)** Intracellular localization after 24-h uptake of (**C**) conventional liposomes and (**D**) immunoliposomes (green), co-stained with LysoTracker (red) and DAPI (blue). Both formulations show diffuse uptake and exhibit lysosomal colocalization.

CONCLUSION

This research successfully established an optimized microfluidic method for fabricating PEGylated immunoliposomes, achieving high EE% and producing highly uniform particles. The formulation provided significant proteolytic protection against enzymatic degradation and maintained excellent physicochemical stability (size, PDI, and EE%) over 60 days. Furthermore, we successfully optimized the surface modification, achieving highly efficient anti-ITGA7 antibody functionalization. Crucially, functionalization accelerated cellular internalization, resulted in pronounced membrane interaction and exhibited lysosomal colocalization. Together, these findings highlight the robust potential of this targeted microfluidic liposomal system as a promising delivery strategy for ERT in Fabry disease.

[1] Azevedo et al., International Journal of Molecular Sciences, 2021;
[2] Yuasa et al., Journal of Echocardiography, 2017;
[3] Di et al., Advanced Drug Delivery Reviews, 2020;

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (#121674/2024-0). INCT NanoFarma: Conselho Nacional de Desenvolvimento Científico e Tecnológico (#465687/2014-8; # #408769/2024-6, #421255/2025-0) and Fundação de Amparo à Pesquisa do Estado de São Paulo (#2014/50928-2; #2025/26972-6; #2025/21457-6; #2025/01386-7)