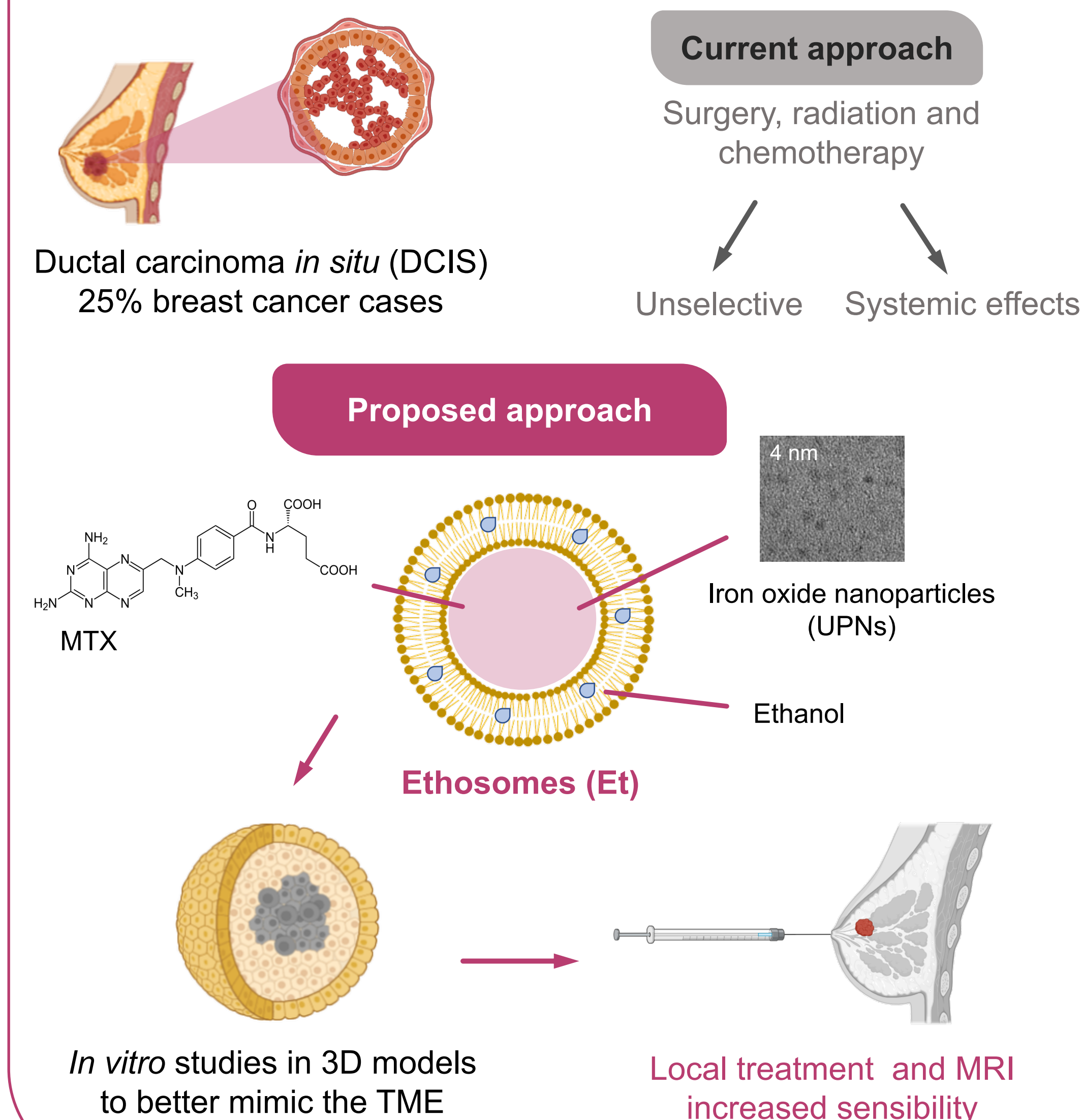
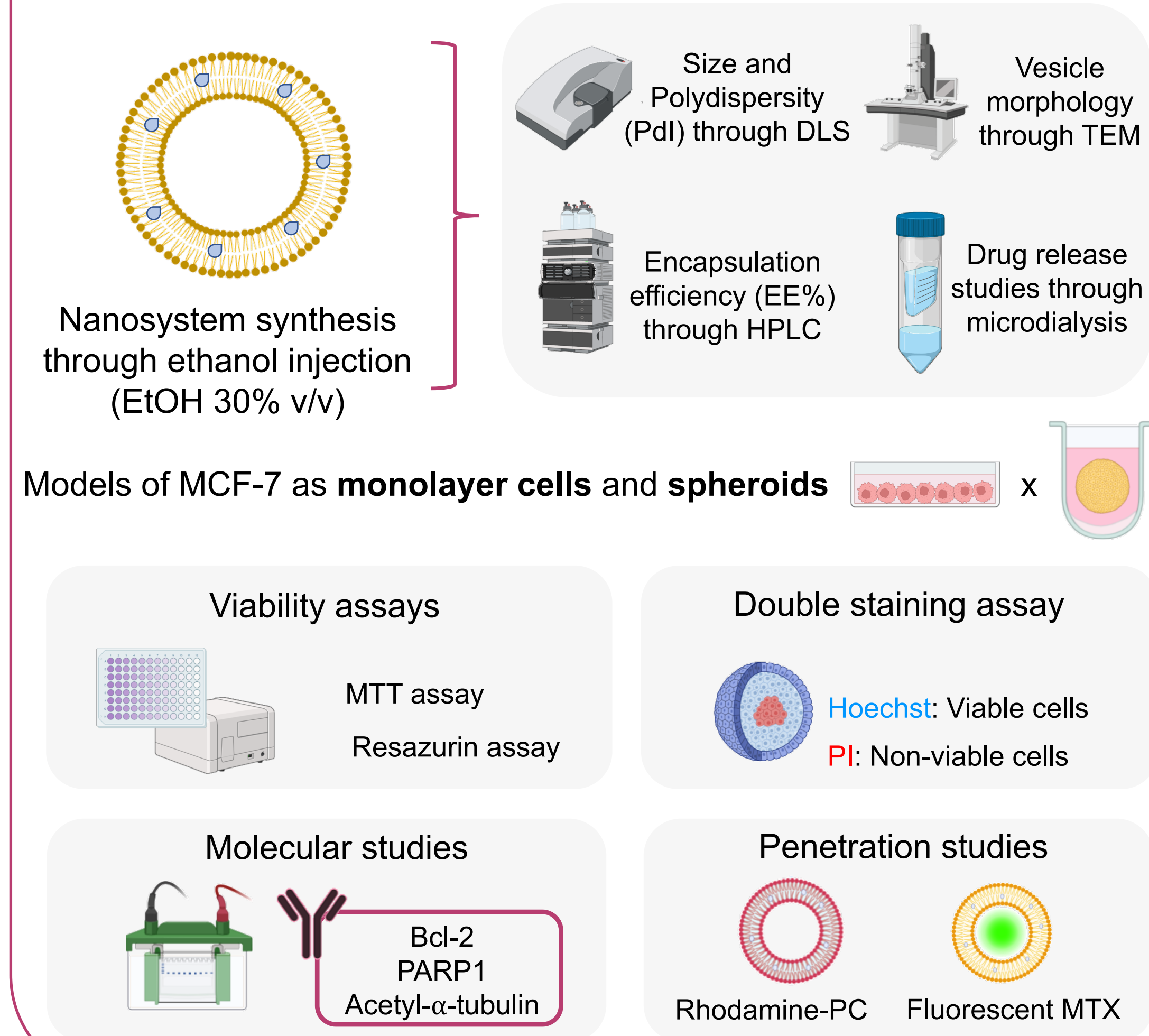


Introduction and aims



Methods



Conclusion

Viability assay findings suggest a more pronounced cytotoxicity effect in spheroids mediated by the co-encapsulation of MTX and UPN into ethosomes, compared to the MTX solution, but not in monolayer cells. Looking at the Western Blot results, the monolayer model displayed more statistically significant results across treatments, in contrast to very few significant differences in spheroids, demonstrating the importance of the employed model. In this sense, the consistently increase in Acetyl-α-tubulin expression after Et MTX+UPN treatment in both models indicates an increased cytotoxicity promoted by the co-encapsulation and thus the presence of UPN, which must be further investigated. Alongside spheroid results, penetration studies findings suggest that increased cytotoxicity in spheroids may be caused by an improved delivery mediated by ethosomes.

Acknowledgements

Results

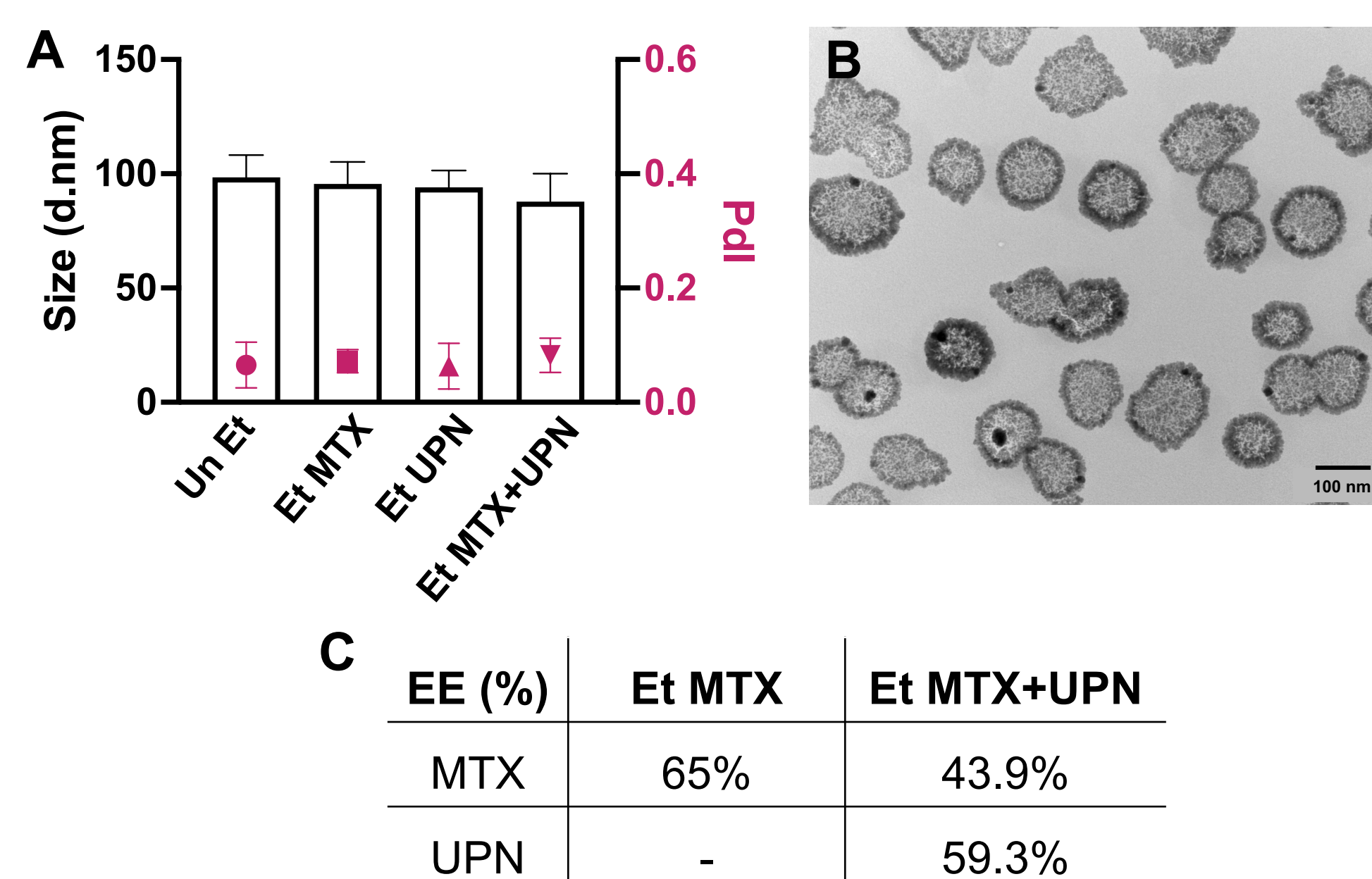


Fig 1. Nanosystem characterization. (A) Measured sizes and polydispersity index (Pdl) of all synthesized formulations using dynamic light scattering (DLS). Size values are shown in d.nm. Results are shown as mean $\pm$ SD ($n > 10$). (B) Unloaded ethosomes image obtained through TEM. Bar scale = 100 nm. (C) Encapsulation efficiency (EE%) of MTX and UPN when single and coencapsulated.

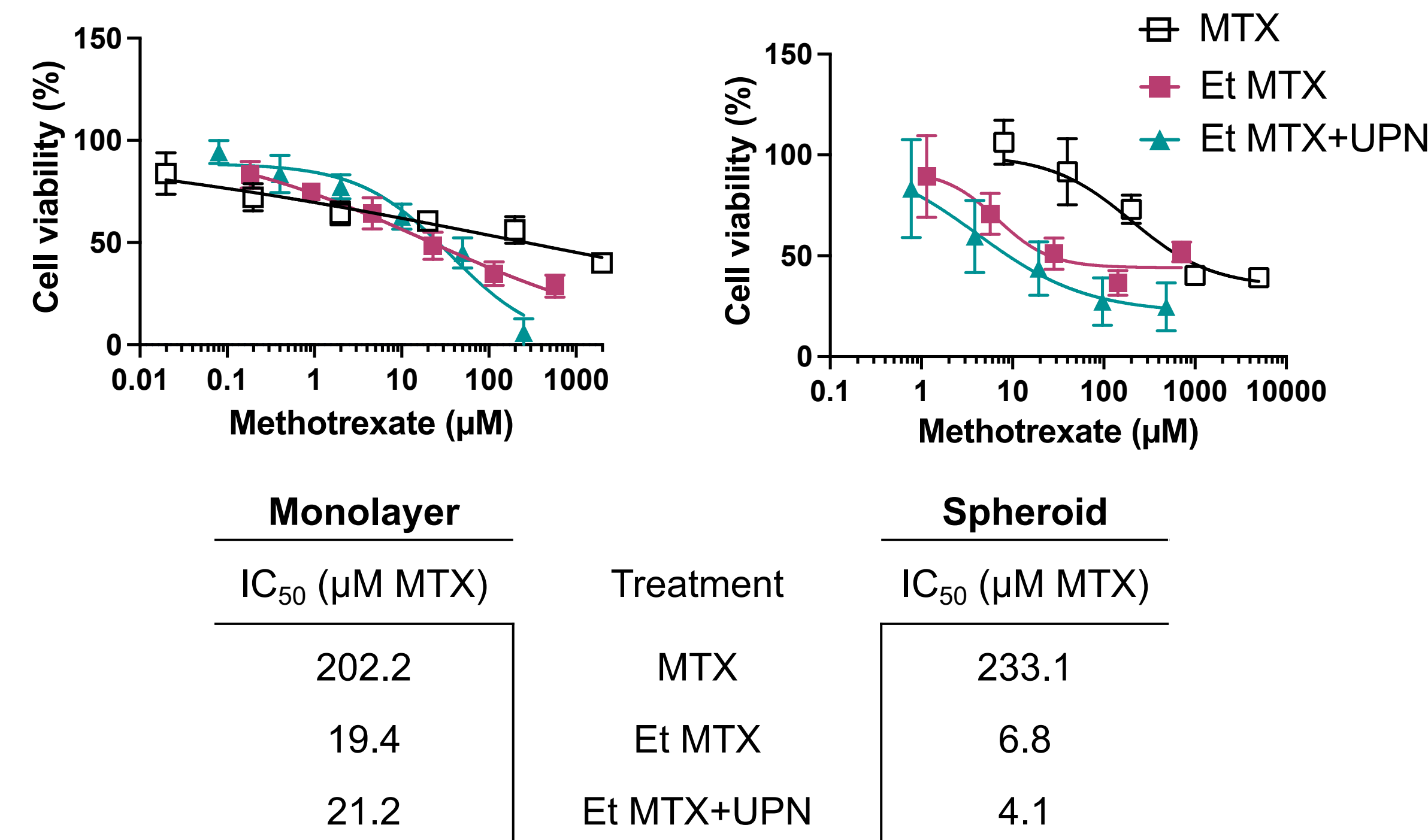


Fig 3. MCF-7 cells viability assays. Dose-response curves and IC₅₀ values after treatment of MCF-7 in monolayer (left) and spheroids (right) with theranostic nanosystem, quantified through MTT and resazurin assays, respectively. Results are shown as mean $\pm$ SD ($n = 4$). Methotrexate concentrations are shown in μM of drug.

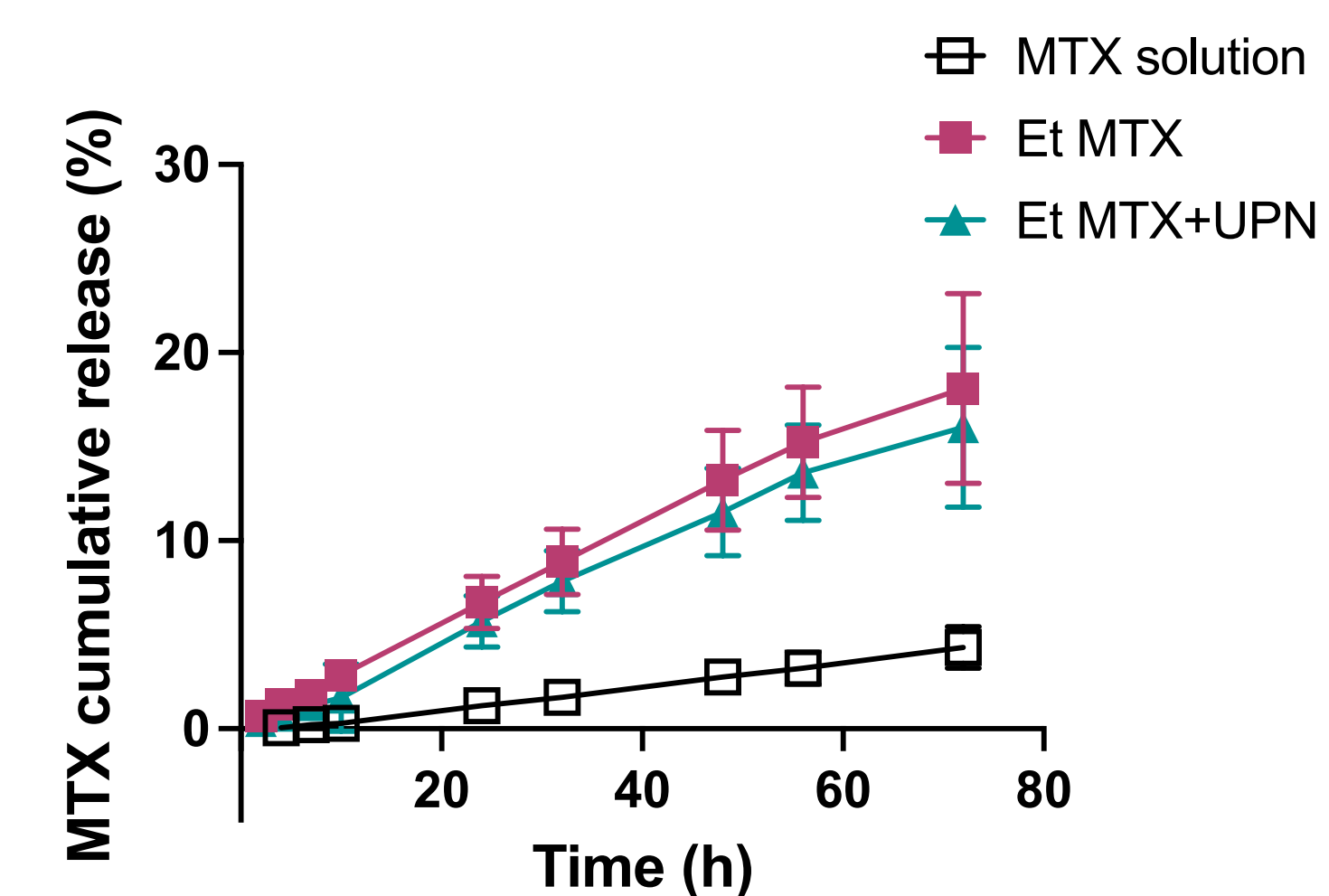


Fig 2. In vitro drug release studies. MTX release as a function of time (2-72 h). A solution of MTX was employed as a control for diffusion across the membrane. Time is presented in hours (h). MTX was quantified through HPLC.

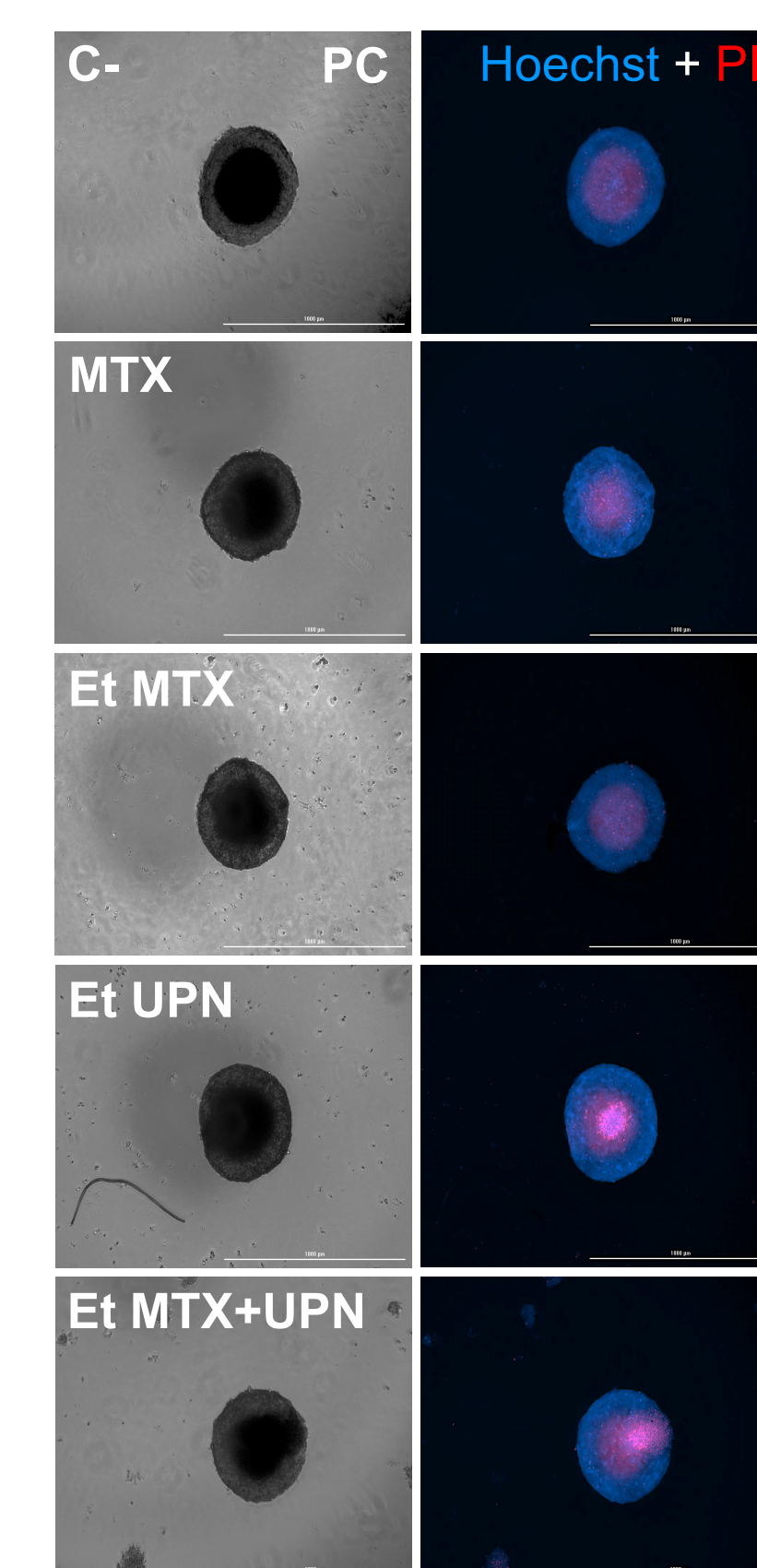


Fig 4. UPN seems to enhance cytotoxicity. To corroborate resazurin assay results, MCF-7 spheroids were dyed with Hoechst (viable cells) and PI (non-viable cells) after 48 h treatment under IC₅₀ concentrations of each treatment group and images were obtained in Phase Contrast, DAPI and RFP filters. Scale bar = 1,000 μm.

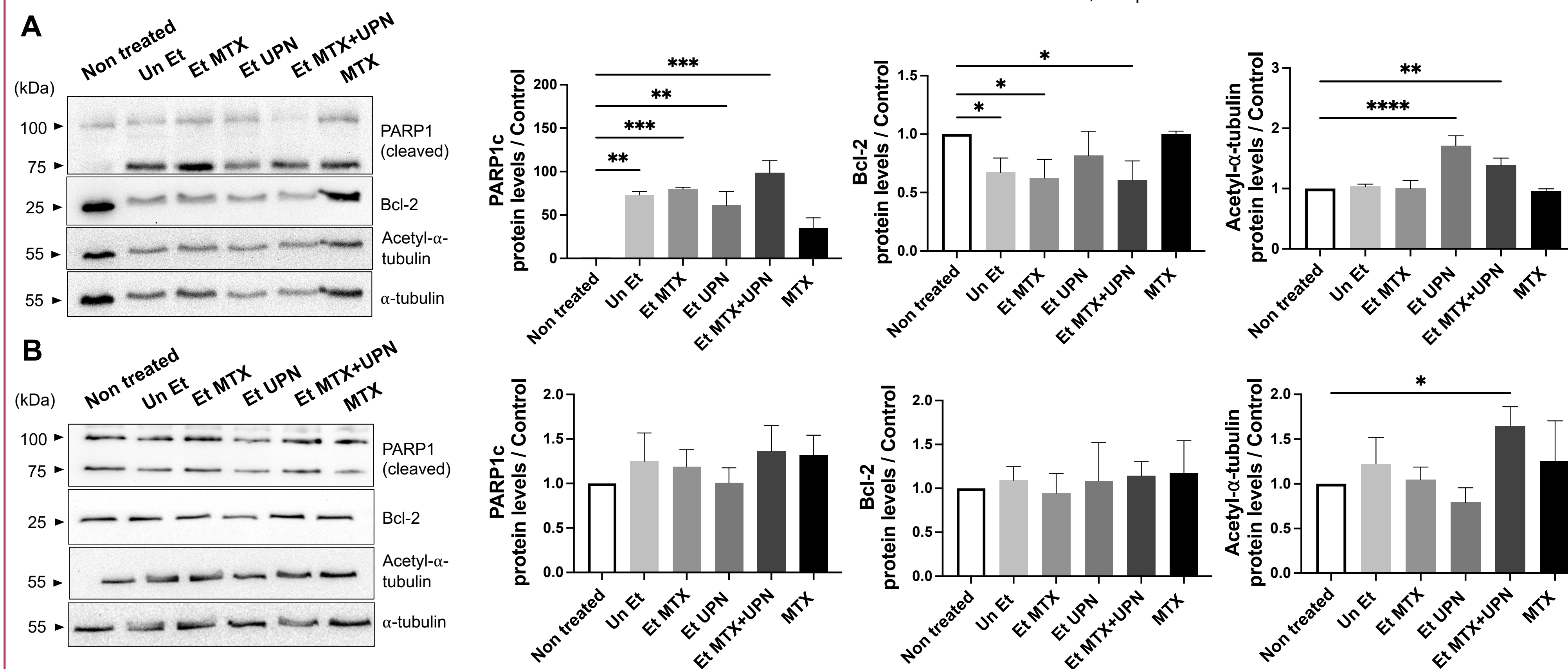


Fig 5. Encapsulation impacts molecular pathways differently across cell models. Molecular studies performed through Western Blot assay in MCF-7 (A) monolayer and (B) spheroids after treatment under IC₃₀ concentrations of each presented group of treatment to investigate whether coencapsulation alters methotrexate pharmacodynamic. α-tubulin was selected as loading control. All the obtained values for treated samples were normalized using its untreated control. Mean $\pm$ SD ($n = 4$, One-way ANOVA). (*) $p < 0.05$ (**) $p < 0.01$ (***) $p < 0.001$ (****) $p < 0.0001$.

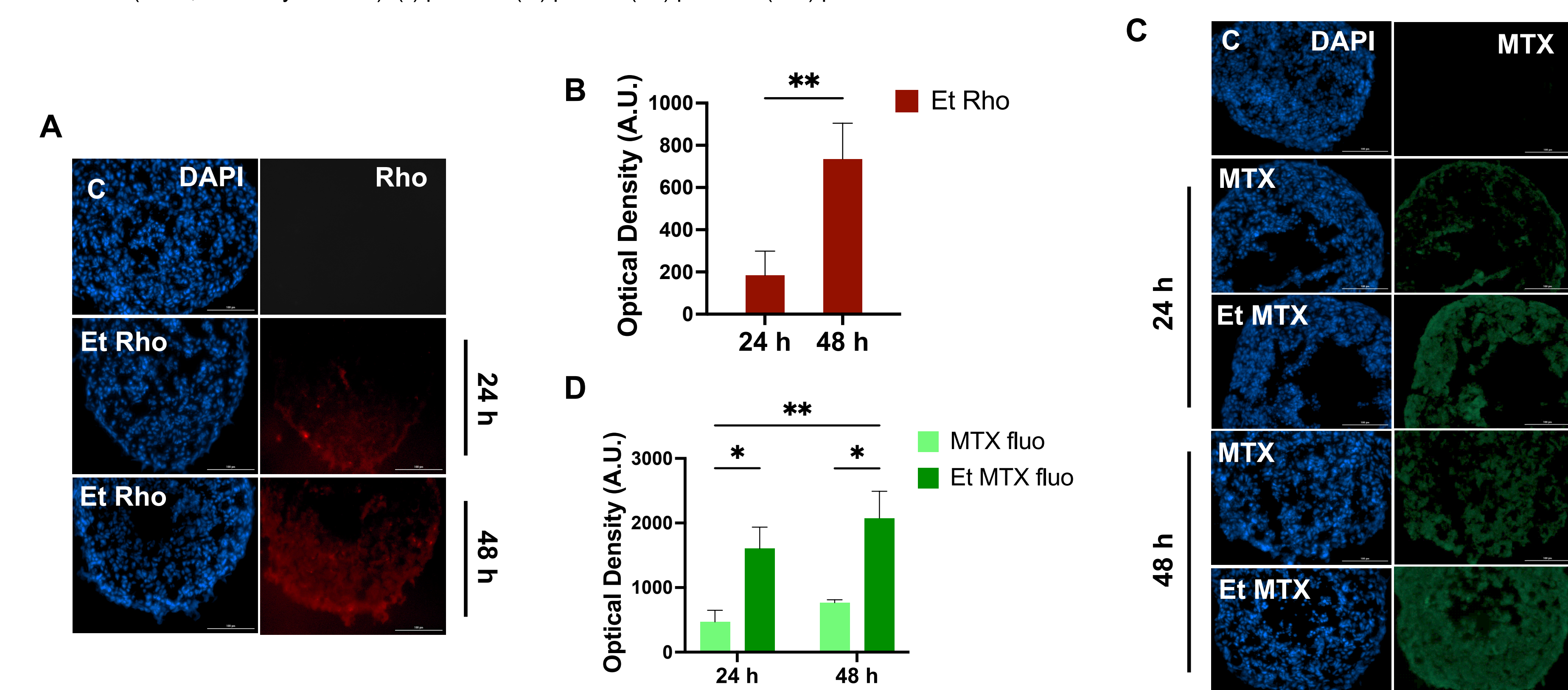


Fig 6. Encapsulation enhances MTX delivery. Penetration studies were conducted to assess nanosystem capacity of penetrating spheroid inner layers. MCF-7 spheroids were sectioned after 24 and 48 h treatment under IC₃₀ of each treatment group and fluorescent signal analysis were conducted. (A) Sectioned spheroids after Et obtained with rhodamine-labeled phosphatidylcholine (Et Rho) treatment. (B) Rhodamine fluorescent signal analysis ($n = 3$; t test). (C) Sectioned spheroids after Et fluorescent MTX treatment. (D) MTX fluorescent signal analysis ($n = 2$; 2-way ANOVA). Images were obtained in DAPI, RFP and GFP filters. Fluorescent signal is shown as optical density as arbitrary unit (A.U.). Mean $\pm$ SD. (*) $p < 0.05$ (**) $p < 0.01$. Scale bar = 100 μm.