Engineered natural nanoparticles for selective drug delivery to the tumor microenvironment through extra domain B-containing (ED-B) fibronectin targeting

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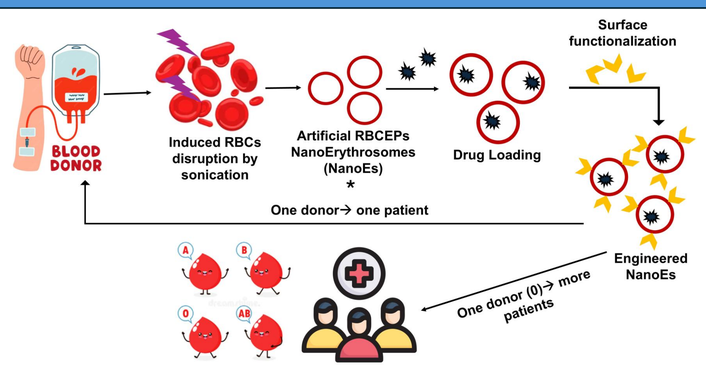
BACKGROUND AND AIM OF THE PROJECT



Red blood cell-derived extracellular vesicles (RBCEVs) possess many excellent features that make them a simple and efficient platform for drug delivery: (i) they can be easily and cheaply produced on a large scale, (ii) they have unknowingly been transfused, for decades, together with RBCs and (iii) they could be used in an allogenic setting (blood group system) thereby eliminating the immunological responses associated with the use of synthetic nanoparticles. The role of the extracellular matrix (ECM) in tumor growth and progression is gaining increasing interest as many ECM proteins are known to be overexpressed in the tumor microenvironment, hence representing an ideal therapeutic target. Over the past two decades, the role of fibronectin (FN) in cancer has been recognized, and FN-targeting strategies have been conceived as promising anti-cancer approaches. One isoform, termed extra domain B-FN (ED-B), also known as "oncofetal" FN, represents one of the most investigated variants for tumor-targeting strategies. ED-B FN has been demonstrated to play a key role in many cancer-related processes, such as tumorigenesis, angiogenesis, metastasis formation, and epithelial-to-mesenchymal transition.

The aim of our project is the engineering of artificial RBC-derived vesicles (Nanoerythrosomes, NanoEs) to obtain a targetable drug delivery system able to recognize ED-B FN.

EXPERIMENTAL PLAN

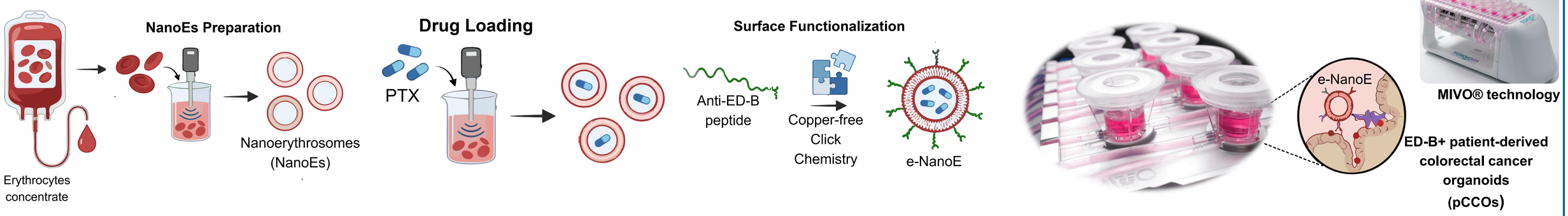


Figure 1. Overview of the project workflow: Nanoerythrosome (NanoE) generation by sonication, followed by Paclitaxel loading via sonication and surface functionalization with the anti-ED-B fibronectin peptide by copper-free click chemistry. e-NanoE therapeutic effect is evaluated through functional *in vitro* assays on patient-derived colorectal cancer organoids (pCCOs) in dynamic conditions (dynamic flow).

ENGINEERING AND CHARACTERIZATION OF NANOERYTHROSOMES (NanoEs)

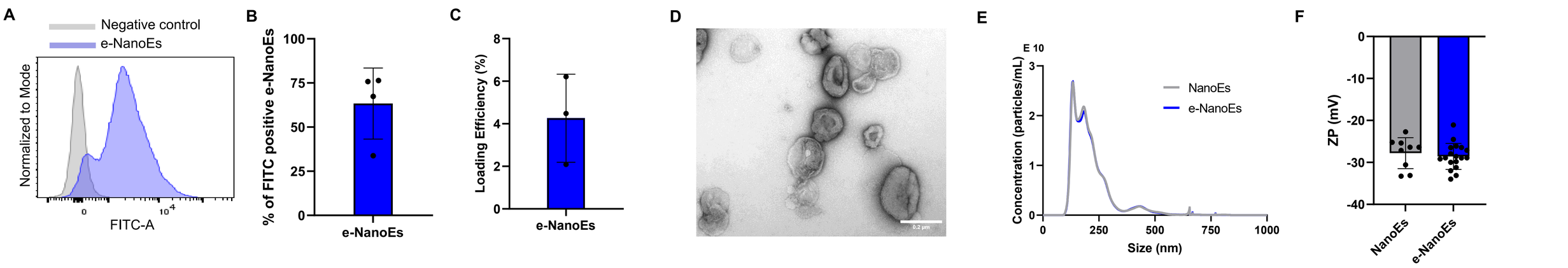


Figure 2. Engineered NanoE (e-NanoEs) production. (A) Representative flow cytometry of e-NanoEs.. Area under gray line identifies the negative control (NanoEs + anti-EDB-FITC-peptide); area under green line represents e-NanoEs (NanoEs + DBCO + anti-EDB-FITC-peptide). **(B)** Percentage of FITC-positive e-NanoEs. **(C)** Paclitaxel loading efficiency determined by high-performance liquid chromatography-mass spectrometry (HPLC-MS). **(D)** Representative transmission electron microscopy (TEM) image of e-NanoEs (scale bar: 0.2um). **(E)** NanoE (grey line, N=3) and e-NanoE (blue line, N=3) particle size distribution determined by nanoparticle tracking analysis (NTA). **(F)** Histogram showing the zeta potential (ZP) of NanoEs (grey column) and e-NanoEs (blue column).

TARGETING, INTERNALIZATION AND THERAPEUTIC EFFICACY OF e-NanoEs IN PATIENT-DERIVED COLORECTAL CANCER ORGANOIDS (pCCOs)

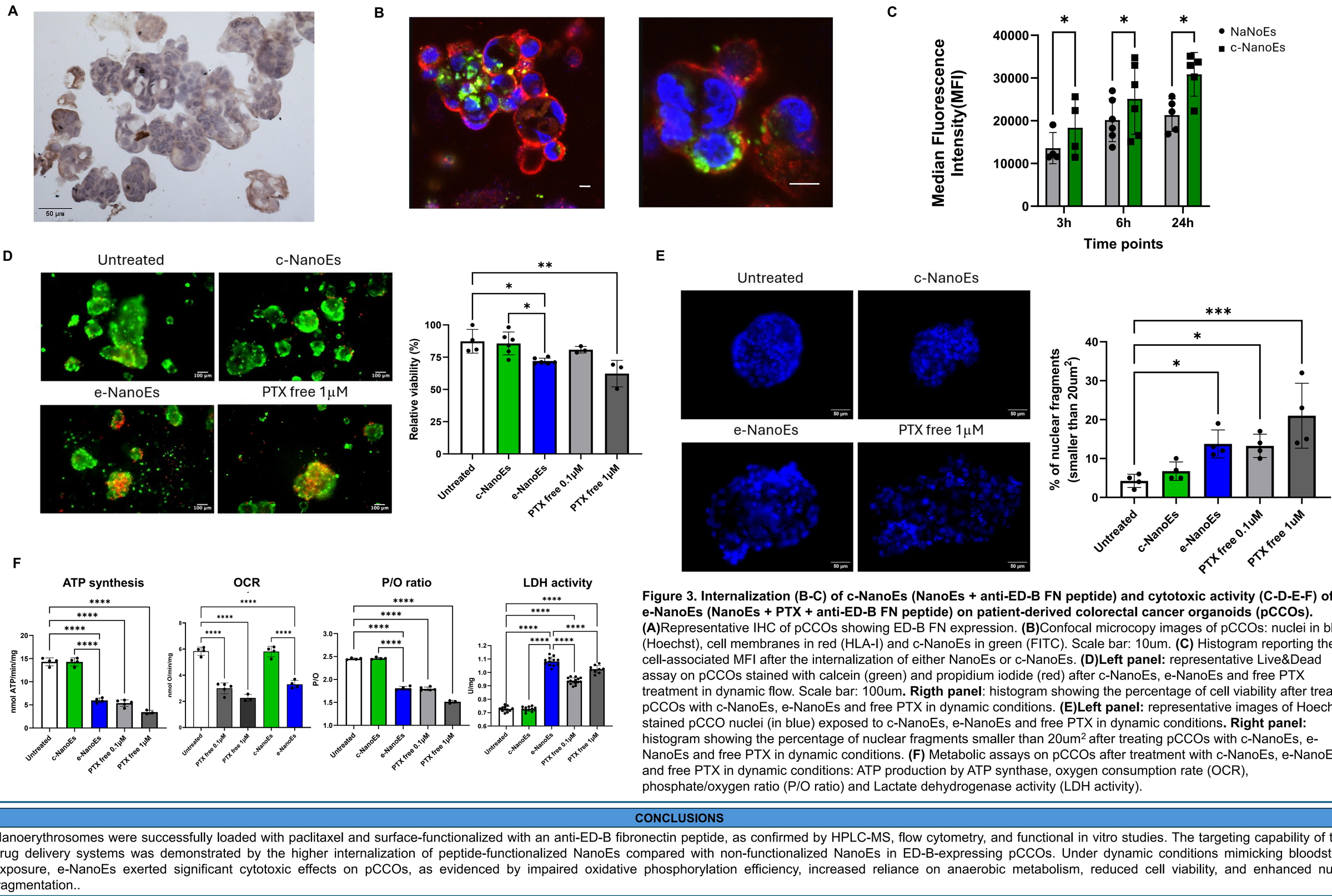


Figure 3. Internalization (B-C) of c-NanoEs (NanoEs + anti-ED-B FN peptide) and cytotoxic activity (C-D-E-F) of e-NanoEs (NanoEs + PTX + anti-ED-B FN peptide) on patient-derived colorectal cancer organoids (pCCOs). **(A)**Representative IHC of pCCOs showing ED-B FN expression. **(B)**Confocal microcopy images of pCCOs: nuclei in blue (Hoechst), cell membranes in red (HLA-I) and c-NanoEs in green (FITC). Scale bar: 10um. **(C)** Histogram reporting the cell-associated MFI after the internalization of either NanoEs or c-NanoEs. **(D)Left panel:** representative Live&Dead assay on pCCOs stained with calcein (green) and propidium iodide (red) after c-NanoEs, e-NanoEs and free PTX treatment in dynamic flow. Scale bar: 100um**. Rigth panel**: histogram showing the percentage of cell viability after treating pCCOs with c-NanoEs, e-NanoEs and free PTX in dynamic conditions. **(E)Left panel:** representative images of Hoechst stained pCCO nuclei (in blue) exposed to c-NanoEs, e-NanoEs and free PTX in dynamic conditions**. Right panel:** histogram showing the percentage of nuclear fragments smaller than 20um^2 after treating pCCOs with c-NanoEs, e-NanoEs and free PTX in dynamic conditions. **(F)** Metabolic assays on pCCOs after treatment with c-NanoEs, e-NanoEs and free PTX in dynamic conditions: ATP production by ATP synthase, oxygen consumption rate (OCR), phosphate/oxygen ratio (P/O ratio) and Lactate dehydrogenase activity (LDH activity).

CONCLUSIONS

Nanoerythrosomes were successfully loaded with paclitaxel and surface-functionalized with an anti-ED-B fibronectin peptide, as confirmed by HPLC-MS, flow cytometry, and functional in vitro studies. The targeting capability of these drug delivery systems was demonstrated by the higher internalization of peptide-functionalized NanoEs compared with non-functionalized NanoEs in ED-B-expressing pCCOs. Under dynamic conditions mimicking bloodstream exposure, e-NanoEs exerted significant cytotoxic effects on pCCOs, as evidenced by impaired oxidative phosphorylation efficiency, increased reliance on anaerobic metabolism, reduced cell viability, and enhanced nuclear fragmentation..