Optimizing Elastin-Collagen Peptide Nanoparticle Fluorescent Labeling for Improved Particle-Organelle Colocalization using Super-Resolution Microscopy

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INTRODUCTION: Osteoarthritis (OA) is a debilitating joint disease affecting millions of individuals worldwide. The disease, characterized by the progressive degeneration of articular cartilage, leads to decreased patient quality of life due to ever-present joint pain and chronic inflammation. Most common in the knee, OA risk is significantly increased for women, the elderly, obese individuals and those having experienced traumatic joint injury. At present, no disease-modifying treatments exist to alter or prevent disease progression. Widely-leveraged OA therapeutics focus primarily on alleviating the perception of pain, using pharmacotherapies such as NSAIDs and COX-2 inhibitors, or IA injection of corticosteroids, hyaluronic acid or platelet-rich plasma. Systemically delivered therapies may never enter the joint space, while, due to rapid clearance from the joint and lack of drug-targeting ability, the majority of injected therapeutics leave the joint compartment entering systemic circulation within a short time - on the order of hours to days. While relief in arthroses is important, current therapies are insufficient, failing to modify the physiology of the disease, often necessitating life-long pain management. Given the lack of therapeutics that inhibit OA progression, the need persists for a targeted treatment option that interrupts the mechanisms involved in disease development. Our elastin-collagen nanovesicle (ECnV) nanodrug delivery platform, developed from self-assembling conjugates of short elastin-like peptides (ELPs) and short collagen-like-peptides (CLPs), addresses this challenge due to their ability to i) bind to damaged collagen, found in abundance in arthritic joints and ii) controllably release their encapsulated cargo/therapeutics. While early findings have demonstrated that 'chondrocyte-like' ATDC5 cells appear to interact with/internalize ECnVs, their mechanism(s) of uptake and intracellular trafficking remain unknown. In this work, we investigate the intracellular trafficking and fate of our ECnVs in ATDC5 cells.

METHODS: Direct visualization of cell-ECnV interactions at the single particle-scale has not previously been attempted. After self-assembly, the mean hydrodynamic diameter of our ECnVs as measured using nanoparticle tracking analysis (NTA), is ~100nm. Particles of this size lie below the diffraction limit of conventional light/confocal microscopy (ca. ~200nm). Thus, to visualize individual cell-ECnV interactions, super-resolution microscopy approaches are required. For this, we turned to STimulated Emission Depletion (STED), a super-resolution technique in which a second 'depletion' laser selectively depletes fluorophore excitation at the periphery of a standard confocal illumination volume (i.e., its point spread function), resulting in a smaller focal point and resolutions approaching ~50nm. Fluorophores suitable for STED must meet the requirements be both bright (have high molar extinction coefficient & fluorescence quantum yield) and highly photostable. Following optimization of fluorophore selection, ATDC5 cells were seeded in 35mm glass bottom dishes at 10,000 cells/cm^2 and cultured overnight. To gain insight into particle uptake, cells were incubated with either 1E9 particles/mL or 1E8 particles/mL of fluorescently labeled (empty-) ECnVs (100- and 1000-fold dilutions of ECnV stock, respectively) for 24 hours. After washing, cells were stained with the nuclear marker Hoescht and flow cytometric analysis of the populations was conducted. Additionally, we evaluated colocalization of ECnVs with early endosome using the CellLight Early Endosome marker (ThermoFisher), an insect baculovirus transduction system (BacMam2.0) that labels early endosomes in mammalian cells using GFP coupled to Rab5a. In short, ATDC5 cells were seeded as above and cultured for 24 hours, after which ~20 viral particles per cell were added to the culture and incubated for 16 hours. After washing, ECnVs were added to the culture at 1E9 particles/mL and incubated for 24 hours before fixation with 4% paraformaldehyde. All samples were then visualized using the Leica Stellaris 8/TauSTED Super-resolution Confocal System.

RESULTS: As a result of our in vitro imaging/colocalization study needs, the use of a far-red shifted fluorophore for ECnV visualization is preferred. While options for far-red/near-IR fluorescent dyes are somewhat limited, we evaluated both AZ647 (a Cy5 derivative) and ATTO594, demonstrating the ability to label our self-assembled ECnVs with both fluorophores while achieving reliable particle sizes and concentrations. Though their molar extinction coefficients are on the same order of magnitude, the quantum yield of AZ647 is a mere 33% compared to that of ATTO594 at 85%. Thus, ECnVs labeled with ATTO594 exhibit far greater brightness and photostability under both confocal and super-resolution imaging techniques. ATDC5 cells incubated with ATTO594 ECnVs were imaged via STED following fixation, demonstrating the novel ability to interrogate cell-single particle interactions **(Figure 1)**. Additionally, flow cytometric analysis of cellular uptake of ATTO594 ECnVs indicates that nearly ~100% of cells interacted with/endocytosed ECnVs (i.e., were ATTO594$^+$) when added at 1E9 ECnV particles/mL. At 1E8 particles/mL only ~16% of cells were ATTO594$^+$ **(Figure 2)**. Since all major forms of endocytosis involve early endosomal cargo encapsulation, we investigated the propensity of internalized ECnVs to colocalize with early endosomes. Confocal and STED microscopy indicated considerable signal overlap between the ATTO594-labeled ECnVs and early endosomes (labeled with GFP fused to Rab5a; **Figure 3)** .

DISCUSSION: Investigation of ECnV-cellular interaction, with resolution at the single-nanoparticle level in chondrocyte-like ATDC5 cells unlocks the ability to investigate both cell uptake and the intracellular trafficking and fate of our novel collagen-targeting ECnV drug delivery platform. The successful formulation of ATTO594 ECnVs, exhibiting superior fluorescent brightness and photostability, enabled dose-dependent study of uptake in a disease-relevant cell type using efficient and high-throughput flow cytometric analyses. Additionally, the colocalization of our particles with early endosomal marker GFP-Rab5a confirms that our ECnVs undergo active uptake by ATDC5 cells, as all major endocytic mechanisms utilize early endosomes in the initial sorting of cargo. Ongoing experiments are underway to elucidate the primary endocytic pathway(s) through which our ECnVs are internalized and trafficked.

CLINICAL RELEVANCE: Insight into the intracellular trafficking and fate of our nanodrug carriers will allow for their rational modifications in order to appropriately deliver varying therapeutic payloads, including nucleic acids and proteins in addition to small-molecule drugs. Expansion of this work will address key barriers in the treatment of OA by merging mechanistic insight regarding ECnV-cell interaction with, ultimately, the optimization of our nanodrug carrier system to precisely (and better) target aberrant cellular signaling within cartilage.

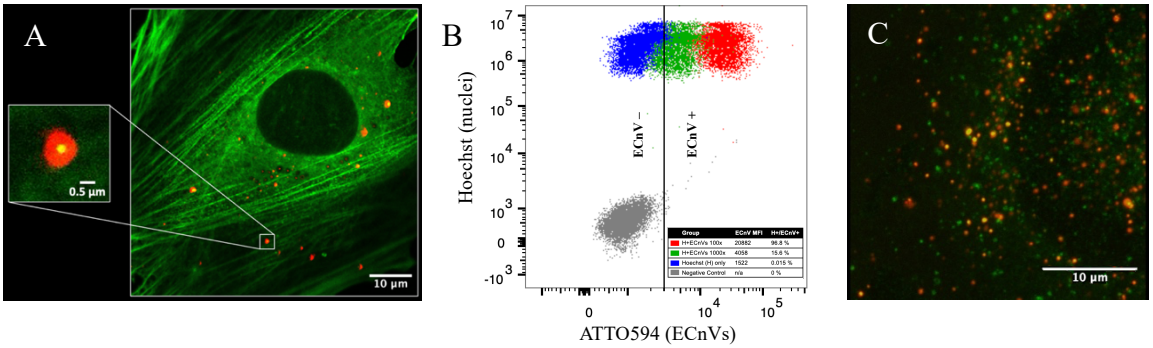

Figure 1: (A) ATDC5 cells and internalized ATTO594 ECnVs; Green – F-actin; Red – ECnVs (confocal); Yellow – ECnVs (STED). **(B)** Flow cytometric analysis demonstrating ~100% ECnV uptake for cells dosed with 1E9 particles/mL concentration. **(C)** Colocalization (yellow) of GFP-tagged early endosomes (Rab5a; green) with ATTO594 ECnVs (red).