ELASTIN COLLAGEN NANOVESICLES – A NOVEL PLATFORM FOR COLLAGEN-TARGETING AND CONTROLLED DRUG DELIVERY

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INTRODUCTION: Joint injuries (*e.g.*, intra-articular fractures & ligament tears) cause joint degeneration and post-traumatic osteoarthritis (PTOA) in ~50% of patients. Accounting for ~12% of OA cases, PTOA places a large burden on patients due to its early onset and impact on quality of life. While injured joint functionality can be restored via surgery, mitigating subsequent PTOA progression remains an unrealized challenge. Currently, clinical treatments focus on pain management, through the use of systemic drugs, but these pose significant concerns regarding off-target interactions/side effects. To overcome challenges associated with systemic drug exposure and to enhance treatment efficacy, intra-articular (i.a.) drug delivery is used. However, the effectiveness of i.a. delivery remains limited due to short i.a. drug retention time and poor drug targeting to cartilage's dense ECM. Thus, a need exists for drug delivery platforms that can target and localize disease-modifying drugs within the joint space and achieve controllable drug release, thereby enhancing drug retention time and reducing toxicity. Our team has shown that conjugation of short elastin-like peptides (ELPs) to short collagen-like peptides (CLPs) allows for the self-assembly of elastin-collagen nanovesicles (ECnVs) that can 1) actively target and bind damaged collagens—as found in injured joints; and 2) exhibit tunable, ELP sequence specific drug loading and (passive and mild hypothermal-triggered) release behaviors. Such attributes should enhance the targeted delivery and prolonged/controlled retention of disease-modifying therapeutic agents in injured joints/cartilage **(Fig. 1A)**. However, the *in vivo* targeting, distribution, retention, and safety of ECnVs has yet to be fully established. In this study, we leveraged multiscale bioimaging approaches (*e.g.*, *in vivo*, epi-fluorescent, confocal, and super-resolution imaging) to characterize the retention and localization of fluorescently-labeled ECnVs (far-red excitation-emission) within intra-, peri- & extra-articular tissues of the healthy/naïve adult mouse joints following i.a. injection.

METHODS: ELPs [(VPGWG)$_6$G'; termed W_6] and CLPs [(GPO)$_8$GG; termed G_8), were synthesized via solid-phase peptide synthesis, conjugated using click chemistry, and purified via HPLC. Tryptophan (W) was chosen for the ELP guest residue due its ability to form strong intra/intermolecular hydrophobic, π-π stacking, and hydrogen bonding interactions, which affect drug loading and morphology. Self-assembly of ELP-CLP conjugates into ECnVs was confirmed via DLS and TEM. After assembly, ECnVs were surface labeled with far-red fluorophores (e.g., AZ647) for *in vitro*, *in vivo* and *ex vivo* visualization. (Empty-)W_6G_8 AZ647-tagged ECnVs were characterized and visualized *in vivo* following an IACUC-approved murine i.a. injection protocol. A ~2mm incision ~10mm medial to the patella was made in the right knees of 12-week-old male C57BL/6 mice (n=12), which were selected based on their accelerated development of post-traumatic joint degeneration following preclinical injury. Female mice will be incorporated into future studies of potential sex-dependent responses. 6uL of labeled ECnVs (3 x 10^{11} particles/mL in saline) were i.a. injected using a micro-liter syringe. Contralateral joints served as shams. Mice were transferred to a UVP *in vivo* imager for *i.a.* far-red fluorescence quantification immediately after injection (0-hr), at 2, 4, 6, 8, 24-hr, and daily until 14 days. Mice were euthanized at 7 or 14-days post injection and the joints harvested for multi-scale imaging. Three-dimensional (3D) imaging of intra- and peri-articular nanoparticle distribution is improved by optical tissue clearing. Several aqueous and non-aqueous clearing approaches, such as thiodiethanol (TDE), tetrahydrofuran-dibenzyl ether (THF-DBE), benzyl alcohol-benzyl benzoate (BABB) and PEGASOS, were assessed for both tissue clearing and nanoparticle/fluorescence preservation. Cleared joints were imaged using spinning disk confocal microscopy, then de-cleared for histology and super-resolution imaging.

RESULTS: W6G8 ELP-CLP conjugates were successfully synthesized and self-assembled into nanovesicles. DLS and TEM analysis indicated average ECnV diameters (D_h) of ~100nm. Epifluorescence microscopy confirmed AZ647 labeling. Following i.a. injection of (empty-)W6G8-AZ647 ECnVs into the right mouse knee joint, robust far-red fluorescence was longitudinally tracked through 14-days **(Fig. 1B)**; no signal was observed in the contralateral (sham) joint. Quantification of *in vivo* fluorescence indicated bi-exponential decay, suggesting a rapid ($t_{1/2}$ ~1.9hours) and subsequent slower ($t_{1/2}$ ~91hours) clearance, with significant signal persisting to 12-days ($p<0.0001$). These data are consistent with complete in-joint retention requiring particle sizes >250nm. Following euthanasia, intact joints were fixed, cleared, and imaged (spinning disk confocal) to assess ECnV survival through optical clearing and to visualize their localization within the intra-/peri-articular space. The current particles showed greatest robustness in TDE, though its low refractive index (RI 1.47) limited imaging depth/quality. THF-DBE (RI 1.56) allowed superior deep-tissue imaging at the expense of significantly reduced fluorescence intensity. Ultimately, we developed a hybrid clearing approach combining THF dehydration and PEGASOS (**THF-BB PEG**; R.I. 1.54) clearing solvents to enable *in situ* far-red visualization at tissue depths >1 mm without compromising fluorescent intensity **(Fig. 1C)**. Confocal imaging indicated ECnV localization to the cartilage surrounding the femoral condyle and extending into the groove, with no evidence of peri-articular distribution.

DISCUSSION: In this study, we synthesized and characterized a fluorophore-tagged (collagen-targeting) drug delivery platform to enhance the delivery and retention of disease-modifying compounds within the injured/early PTOA joint. Few strategies exist that utilize macromolecular peptides that target damaged collagen while offering tunable drug delivery kinetics, which ECnVs provide. Using a combination of longitudinal in vivo imaging, macroscale distribution studies, and high-resolution fluorescent imaging techniques—we tracked labeled ECnVs following intra-articular injection. These approaches allowed us to characterize vesicle behavior across multiple spatial scales—from the whole animal to the cellular and subcellular levels. 3D reconstruction of joints using confocal microscopy, post tissue clearing, provides a qualitative picture of particle distribution, capturing how these vesicles penetrate and persist in joint structure. This data provides early insight into the temporal dynamics of ECnV retention and support their exploration for targeting/sustained release in joint tissues. Future work will investigate empty- and cargo-laden- ECnV biodistribution in healthy vs. injured joints, as well as establish the biosafety and efficacy of ECnVs as a targeted and controllable delivery platform for PTOA therapeutics.

SIGNIFICANCE/CLINICAL RELEVANCE: This study establishes ECnVs as collagen-targeting nanovesicles capable of sustained intra-articular retention, addressing a key barrier to localized PTOA therapy. By characterizing in vivo distribution and clearance dynamics, critical preclinical insight into targeted drug delivery mechanisms is provided, laying the groundwork for translational studies aimed at improving therapeutic efficacy and long-term joint preservation.

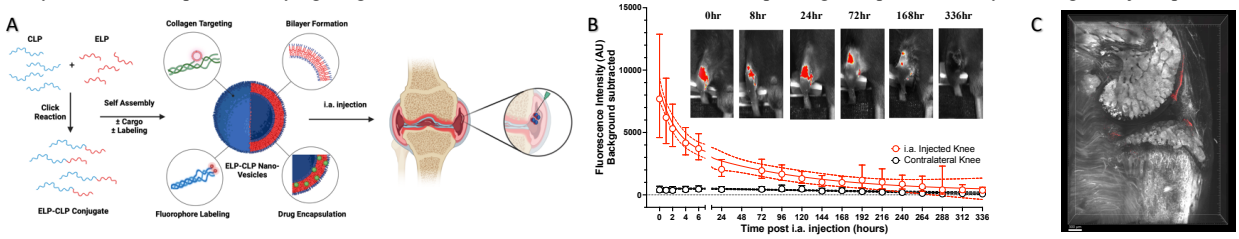

Fig 1: (A) Conjugation of ELP and CLP peptides produce elastin-collagen nanovesicles (ECnVs) that can bind to degraded collagen in osteoarthritic joints and deliver therapeutic cargo. **(B)** Longitudinal *in vivo* tracking of (empty-)W_6G_8-AZ647 ECnVs (red) following i.a. administration to mouse joints, overlaid on visible light images of the mouse body. Quantification of in-joint ECnVs following i.a. injection is shown. **(C)** 3D reconstruction of optically cleared joint showing localized distribution of ECnVs (red) within the intact knee joint.