Integrin targeting enhances uptake of fluorescent silica nanoparticles by chondrocytes in cartilage tissue

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INTRODUCTION: The effectiveness of intra-articular (IA) drug delivery approaches for osteoarthritis (OA) is hindered by the dense, avascular extra-cellular matrix (ECM) of cartilage and the rapid synovial fluid clearance time on the order of hours.[1,2] Engineered particle-based systems for drug delivery have yielded modest improvements in joint residence time compared to free drug,[3,4] but their ability to localize and endure in cartilage or chondrocytes is largely unexplored. Integrins expressed on the surface of chondrocytes mediate mechanical sensing and signaling through binding to the ECM.[5,6] Arg-Gly-Asp (RGD) is a conserved sequence across many matrix components and has been used to improve cellular adhesion in tissue engineered scaffolds via integrin-binding.[5] Microspheres functionalized with RGD motifs have enabled targeting of chondrocytes for cartilage regeneration.[7,8] However, functionalization of cartilage-penetrating drug delivery vehicles with integrin-binding RGD motifs have not been explored. Ultrasmall ($d_h \sim 6$ nm) fluorescent poly(ethylene glycol) coated core-shell silica nanoparticles (Cornell Prime Dots, or C' Dots) are an ultrabright, photostable, fluorescence imaging tool that have demonstrated clinical translatability in oncology applications.[9,10,11,12] Recently, C' Dots were investigated as cartilage-penetrating drug delivery vehicles in cartilage.[13] C' Dots rapidly diffused into healthy articular cartilage ($D \sim 2$ μm^2/s) and were taken up by tissue-resident chondrocytes. Surface functionalization of C' Dots with collagen-II targeting peptides altered transport kinetics at the tissue-level,[14] but cell targeting to enable localization of C' Dots to chondrocytes has not yet been investigated. We hypothesized that RGD modification would enhance chondrocyte uptake of C' Dots. To test this hypothesis, we incubated whole cartilage explants with C' Dots functionalized with 0, 5, 10, or 20 RGD peptides and characterized intracellular fluorescence using confocal microscopy.

METHODS: *C' Dot Synthesis:* Cyanine 5 fluorophore-encapsulating C' Dots were synthesized following previously established methods.[9,10] Post-PEGylation, heterobifunctional PEGs were employed to introduce integrin-binding functionalization via efficient, biorthogonal click chemistry, with resulting experimental groups containing 0, 5, 10, and 20 cyclic RGD (cRGD) peptides per particle.[10] *Cellular Internalization:* Cylindrical cartilage explants (d = 6 mm, h = 2 mm, n = 4-7 per group) were cut from the patellofemoral groove of 1-3 day old bovine stifle joints and randomly assigned to C' Dot groups. C' Dots in media (2 μM) passively diffused through the articular surface and radial edges of submerged explants.[15] After 24 hours, C' Dot plugs were moved to fresh media and washed every other day until imaging. *Imaging:* Cartilage plugs were bisected with a lubricated blade for staining (30 minutes Calcein, AM & Sytox Blue nucleic acid stain). Tissue penetration and chondrocyte internalization of C' Dots were assessed at the bisected plane using a Zeiss 710 inverted confocal microscope and the following excitation wavelengths: Sytox Blue (405 nm), Calcein, AM (461 nm), tissue reflectance (561 nm), and Cyanine-5 (633 nm). *Image Analysis:* A custom MATLAB code created logical masks to isolate C' Dot signal in chondrocytes. Intracellular fluorescence was heterogeneous and organized into vesicular structures. Subcellular vesicles were isolated for analysis of brightness and number detected per cell in the imaged confocal plane. *Statistical Analysis:* Fluorescent intensities were compared at each timepoint using ANOVA and Tukey's post-hoc pairwise comparisons (α = 0.05).

RESULTS: Unmodified and integrin-targeted C' Dots were internalized by chondrocytes after 24 hours **(Fig. 1A)**. Area-normalized C' Dot fluorescence signal decreased over time, following first-order exponential decay ($y = Ae^{-t/\tau} + C$) **(Fig. 1B**, inset**)**. 10 cRGD-PEG-C' Dots exhibited a nearly 2-fold greater initial intensity in chondrocytes (**Fig. 1B,** $p = 0.02$ and 0.01 vs. PEG-C' Dots and 20cRGD-PEG-C' Dots, respectively). 5 cRGD-PEG-C' Dots exhibited the longest decay constant ($\tau = 4.4$ days vs. 3.1 days for PEG-C' Dots). This fluorescent signal was localized to intra-cellular vesicular structures, rather than the cytoplasm. The number of vesicles containing 5 cRGD-PEG-C' Dots or 10 cRGD-PEG-C' Dots decreased over time (**Fig. 1C**, $p < 0.02$). However, fluorescent intensity in isolated vesicles did not change over time for any C' Dot formulation (**Fig. 1D**, $p > 0.05$). For the PEG-C' Dots and 20 cRGD-PEG-C' Dots, the number of vesicles identified per chondrocyte trended towards decreasing over time but this effect was not significant **(Fig. 1D)**.

DISCUSSION: Integrin targeting increased the efficacy of chondrocyte internalization (10 cRGD/particle) and slowed clearance (5 cRGD/particle). These results suggest that cRGD peptides may increase chondrocyte endocytosis via an integrin-mediated mechanism. In other studies, multivalent RGD tracers improved targeting efficiency in other cell types due to bivalent receptor binding and local RGD enrichment at the cell surface.[6] Since the C' Dots present multiple ligands, it is intriguing that an intermediate number of RGD peptides per particle improved the uptake into the chondrocytes but 20 cRGD peptides per particle did not. These observations suggest that internalization of C' Dots was mediated by ligand density at the chondrocyte surface. Fluorescence was localized to subcellular vesicular structures and not observed in the cytoplasm.[16] Interestingly, the number of vesicles detected per cell decreased over time, but the brightness of these vesicles did not. These results suggest that active mechanisms of internalization, rather than passive mechanisms, are responsible for the uptake and retention of integrin-targeted C' Dots in chondrocytes. RGD-functionalized C' Dots may be well suited for drug delivery applications in OA and rheumatoid arthritis, where $\alpha_v\beta_3$ integrins are overexpressed in chondrocytes and synoviocytes.[6,17]

SIGNIFICANCE: Integrin-targeting enhances chondrocyte localization of a cartilage-penetrating drug delivery system for OA therapeutics, which may improve the efficacy and tissue retention of intra-articular therapies.

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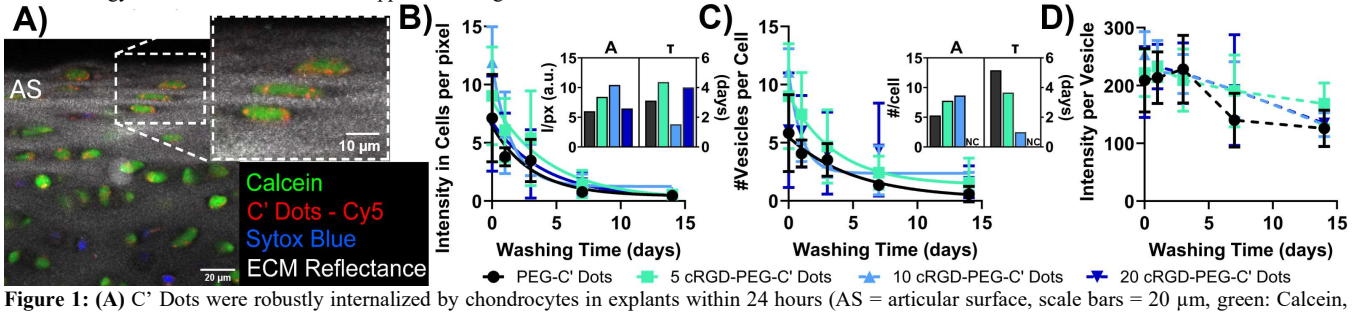

Figure 1: (A) C' Dots were robustly internalized by chondrocytes in explants within 24 hours (AS = articular surface, scale bars = 20 μm, green: Calcein, AM; blue: Sytox Blue; red: C' Dots; grey: tissue reflectance). **(B)** C' Dot fluorescence in chondrocytes decreased during washing (integrated pixel intensity normalized by area). 10 cRGD-PEG-C' Dots exhibited a nearly 2-fold increase in chondrocyte uptake (**inset**, vs. PEG-C'Dots, $p = 0.02$). **(C)** The number of fluorescent vesicles identified per cell decreased during washing. 5 cRGD-PEG-C' Dots and 10 cRGD-PEG-C' Dots exhibited the highest initial vesicle counts (**inset**, $p < 0.02$ vs. PEG-C' Dots). 20 cRGD-PEG-C'Dots did not follow exponential decay (NC = not calculated). **(D)** Fluorescence intensity per vesicle trended towards decreasing during washing, but this effect was not significant ($p > 0.05$). Solid lines **(B,C)**: exponential fits. Dashed lines **(D)**: connect data points for enhanced visibility.