Collagen-derived macropeptides rapidly penetrate articular cartilage and associate with extracellular collagen

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INTRODUCTION: The most abundant extracellular matrix (ECM) macromolecules in articular cartilage are collagen-II, aggrecan, and hyaluronic acid.[1,2] Type II collagen forms a fibrillar meshwork and plays a crucial role in tissue tensile mechanics, as well as binding proteoglycans.[1] The hallmark of osteoarthritis (OA) in cartilage is the loss of proteoglycans and collagen, leading to tissue erosion and loss of mechanical properties.[1,3] The loss of cartilage collagen is an irreversible feature of OA,[3] and as such therapeutic strategies could aim to replenish collagen and augment damaged tissue. Delivery of collagen tripeptides,[1] low MW collagen hydrolysate,[4,5] and atelocollagens[2,6] have shown some evidence of increasing tissue collagen content, however, the effect of collagen supplementation on the mechanics of the underlying fibrillar meshwork has not yet been fully explored. Large (> 300 kDa) collagen macromolecules present a delivery challenge in synovial joints owing to the dense size- and charge-restrictive ECM of articular cartilage.[7] In this study, we investigated a collagen-derived macropeptide preparation (MCOL) as a novel cartilage-penetrating therapeutic candidate with a potential mechanical mechanism of action. We hypothesized that MCOL would diffuse into cartilage within 2 hours (D ~ 20 μm^2/s, based on peptide size[7]) and associate with the existing collagen network. For these studies, we incubated whole cartilage explants with fluorescently labeled MCOL for 15 min to 24 hours and assessed the effect of the underlying collagen network under steady-state conditions.

METHODS: *MCOL preparations:* Collagen macropeptides from bovine skin were fractionated by ultrafiltration and MCOL preparations were formulated in PBS or DMEM and sterile filtered. MCOL macropeptides (nominal MW range ~10 kDa to ~50 kDa) were fluorescently labeled with amine-reactive IVISense 680-NHS ester fluorescent dye. *Explants:* Cylindrical cartilage plugs (diameter: 6 mm for diffusion or 4 mm for steady-state, height: 1 mm, n = 3-6 per condition) were cut from the patellofemoral groove of 1-3 day old bovine stifle joints[8] and randomly assigned to MCOL groups. MCOL in different solutions (PBS for diffusion; supplemented DMEM[8] for steady-state) passively diffused into submerged explants as described previously.[8,9] *Imaging:* After incubation, explants were rinsed in PBS and bisected for confocal microscopy to assess MCOL penetration. For diffusion studies, MCOL fluorescence was recorded utilizing a 633 nm excitation laser. For steady-state studies, multiphoton imaging with an excitation wavelength of 800 nm captured signal from second harmonic generation (SHG) of collagen (400 nm) and MCOL fluorescence (680 nm). *Image Analysis:* The diffusion experiment was modeled as a 1D diffusion problem under the assumption that MCOL only penetrated from the articular surface in the imaged region.[8,9] MCOL fluorescence was averaged in 20 μm slices parallel to the articular surface and normalized using a custom MATLAB code.[8] Data from 0-300 μm were fitted to a 1D diffusion equation derived from Fick's 2nd law to obtain diffusivity, D.[10] For steady-state experiments, MCOL and SHG signals were averaged within 50 μm bins parallel to the surface. *Statistical Analysis:* Diffusion coefficients were compared using ANOVA and Tukey's post-hoc pairwise comparisons in R Studio (α = 0.05). Correlation analyses and ANOVA were performed on SHG and MCOL profiles in MATLAB (α = 0.05).

RESULTS: Fluorescently labeled MCOL was observed within cartilage explants after 15 min incubation. Diffusion profiles stretched deeper into the tissue after 30 and 60 min (**Fig. 1A**). After 3 hours, the system approached steady-state as evidence of a MCOL diffusion profile was no longer confined to the top 50% of the tissue. Diffusion coefficients calculated at 15 min were variable, but coefficients calculated at 30 min or 60 min were not different (**Fig. 1B**, p = 0.26). Thus, 30 min MCOL incubation was used to estimate its transport kinetics (D ~ 4.9 μm^2/s). Collagen SHG signal and MCOL fluorescence were captured concurrently after 60 min and 24 hour culture, revealing a diffusion profile after shorter incubation and a depth-dependent fluorescence profile after steady-state was reached (**Fig. 1C**). SHG signal was not distinguishable between MCOL and control groups. While a diffusion profile was apparent in the top 50% of the explants after 60 min (**Fig. 1D**, top), the MCOL depth-dependent fluorescence profile was strikingly similar to the collagen SHG profile captured after 24 hours (**Fig. 1D**, bottom). In fact, MCOL fluorescence was spatially correlated with collagen SHG after 24 hours (**Fig. 1E**, $R^2 = 0.52$, $p < 0.0001$).

DISCUSSION: Healthy articular cartilage presents the greatest barrier to small molecule transport due to its high aggrecan content and low porosity.[7] MCOL peptides exhibited a diffusivity of 4.9 μm^2/s, 4-fold lower than would be predicted based on its molecular size.[7] This reduced diffusivity is consistent with reversible matrix binding interactions. At steady-state in cultured tissue, the similarities of the MCOL fluorescence and collagen SHG profiles, as well as the correlation of these data, also suggest that MCOL peptides may associate with native collagen (**Fig. 1D-E**). During development, circumferential growth of collagen fibrils and fibers occurs by addition of other fibrils,[3] however, it may be possible for individual collagen macromolecules to bind to existing fibrils and fibers via collagen-collagen interactions, such as lysine-hydroxylysine bonds.[11] Our results suggest that MCOL may associate with extracellular collagen in articular cartilage. While previous studies have proposed a biological effect of low MW collagen peptides or atelocollagens,[1,2,4-6] our data suggests that MCOL could have a mechanical therapeutic effect. In OA cartilage, increased tissue porosity may enhance MCOL transport kinetics and expose more collagen fibrils.[12] MCOL associated with damaged collagen fibrils may thus have the potential to increase fiber diameter and strength in degraded tissue.

SIGNIFICANCE: MCOL rapidly diffused into articular cartilage and associated with native collagen, consistent with collagen binding. MCOL may augment the collagen fibrillar meshwork and have utility as a therapeutic for OA.

REFERENCES: [1]Naraoka+ *Arth Res Ther* 2013; [2]Furuzawa-Carballeda+ *Eur J Clin Inv* 2009; [3]Gottardi+ *PLoS One* 2016; [4]Oesser+ *Cell & Tiss Res* 2003; [5]De Luca+ *J Clin Med* 2019; [6]Suh+ *Tiss Eng Regen Med* 2021; [7]DiDomenico+ *Nat Rev Rheum* 2018; [8]Fortin+ *Acta Biomat* 2025; [9]DiDomenico+ *J Biomech* 2017; [10]Crank *Diffusion* 1975; [11]Antipova+ *PloS One* 2012; [12]DiDomenico+ *J Biomech* 2019.

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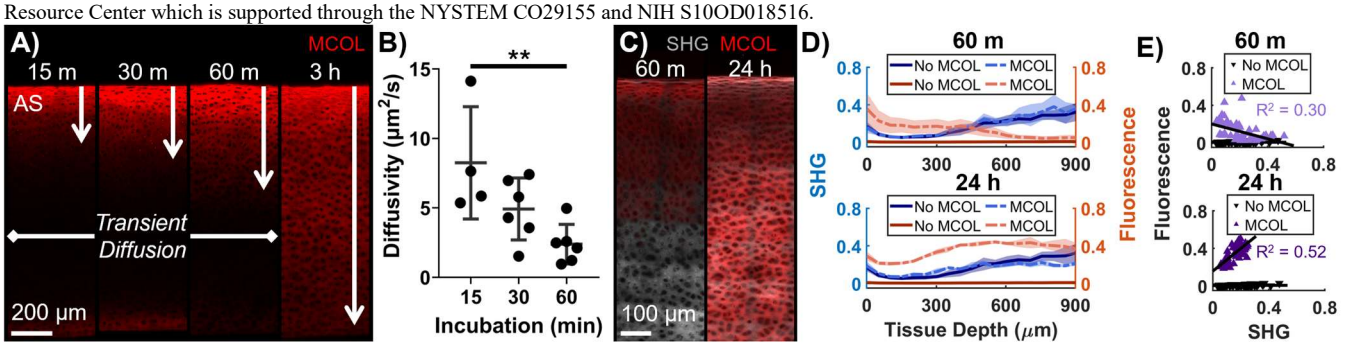

Figure 1: (A) MCOL peptides rapidly diffused into cartilage explants within 1 hour and approached steady-state after 3 hours (AS = articular surface, red: IVISense 680-tagged MCOL). **(B)** MCOL diffusivity was variable for short incubation times whereas the diffusion profile began to flatten after 1 hour (p = 0.006). After 30 min, MCOL diffusivity was estimated to be 4.9 μm^2/s. **(C)** Multiphoton imaging captured collagen signal (SHG) and MCOL fluorescence in cartilage explants simultaneously, visualizing transient diffusion (60 min) and steady-state (24 hours). **(D)** A MCOL diffusion profile was evident after 60 min (top panel). After 24 hours, more MCOL was observed deeper in the tissue and evidence of a diffusion profile was lost (bottom panel). **(E)** Under transient conditions, MCOL was negatively correlated with SHG signal ($p < 0.001$). At steady-state, MCOL was positively correlated with SHG signal ($p < 0.0001$).