

## Synthesis of Dual-Energy CT Contrast Agent for MMP Activity

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**INTRODUCTION:** Osteoarthritis (OA) is a debilitating joint condition characterized by the loss of cartilage tissue. Cartilage is a porous material composed of a type-II collagen fibril network that provides structure and tensile strength, and an anionic glycosaminoglycan (GAG) matrix that retains interstitial water. In early OA, GAGs are depleted from cartilage as a result of cytokine-mediated upregulation of matrix metalloproteinases (MMPs) and downregulation of GAG production. As GAGs are depleted, the increased permeability and decreased charge of the cartilage further exposes chondrocytes to anionic cytokines (e.g., IL-1, TNF- $\alpha$ ), leading to accelerated cartilage degeneration and a cascade of continued damage. OA is a heterogeneous disease instigated by several pathophysiological mechanisms (mechanical insult, metabolic alterations, and/or cell senescence) that generate similar joint pathoanatomy. There is a consistent alteration in cell signaling where the release of inflammatory cytokines induces chondrocyte hypertrophy and the production of MMPs and other pro-inflammatory mediators. This, in turn, stimulates the transformation of synoviocytes to proliferate and release inflammatory cytokines, creating a positive feedback loop that sustains upregulation of MMPs and other catabolic enzymes that catabolize cartilage. As MMPs are key mediators of cartilage degradation and a biomarker for catabolic signaling, they are suitable targets for early OA detection. Previously, we developed a cationic iodinated contrast agent for non-invasively measuring the anionic GAG content of cartilage using contrast enhanced computed tomography imaging (CECT). Dual-energy or spectral CT imaging differentiates materials using attenuation measurements obtained at multiple energy levels. Here we introduce the concept of a dual-energy CT agent based on bromine and iodine, which can be distinguished by measuring attenuation below and above the k-edge of iodine at 33.2 keV. The CT agent design includes an MMP-cleavable peptide covalently linked to a brominated dendron and an iodinated dendron terminated with collagen II binding peptide (CBP), predicated on the hypothesis that when the peptide is cleaved by MMPs, the bromine dendron will diffuse from the cartilage, while the iodine dendron will remain bound to the cartilage matrix through CBP. The relative X-ray attenuation of iodine to bromine, measured by dual energy CT, correlates to MMP activity. As a prelude to this CT agent, we synthesized a generation 1 (G1) iodinated and brominated dendron as well as a functional two-color fluorescent contrast agent to measure MMP activity. This fluorescent contrast agent consists of an MMP-cleavable peptide covalently linked to two different fluorophores. As the two fluorophores participate in FRET, the fluorescent signal is diagnostic of the MMP activity.

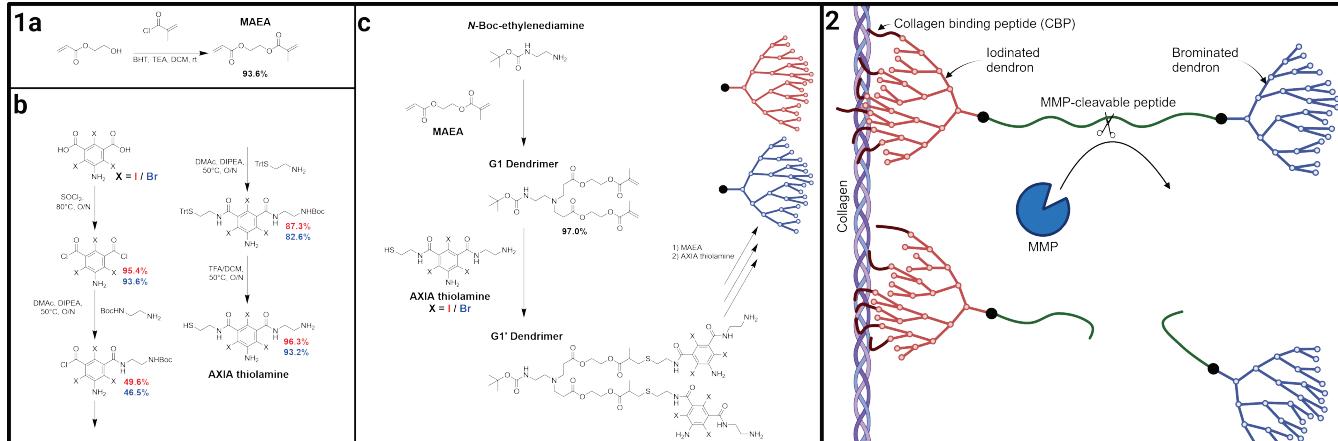
**METHODS:** The brominated and iodinated dendron was formed by alternately adding two kinetically asymmetric monomers, 2-[(methacryloyl)oxy]ethyl acrylate (MAEA) and (2,4,6-trihalo-5-aminoisophthalic acid) AXIA thiolamine, where X is either iodine or bromine. MAEA monomer was synthesized by reacting 2-hydroxyethyl acrylate with methacryloyl chloride (Fig 1a). The AXIA thiolamine monomer was produced from AXIA (Sigma) through conversion of carboxylic acids to acyl chlorides and subsequent reaction with N-Boc-ethylenediamine and Trt-cysteamine (Fig 1b). Starting with N-Boc-ethylenediamine as the dendron core, the acrylate group in MAEA was reacted with the primary amine via Michael addition to form a G1 dendron. AXIA thiolamine was then added through a thiol-ene reaction to complete the first generation, integrating the contrast agent into the dendron structure and providing the primary amine to add subsequent generations (Fig 1c). Reactions were monitored by TLC and product confirmed by <sup>1</sup>H NMR or, in the case of the G1 dendron, HMQC NMR. The cleavage kinetics of an MMP cleavable peptide sequence were assessed using two different fluorophores: CY3 and CY5. A CY5-maleimide linker (Cytiva) was conjugated via a C-terminal cysteine to a peptide containing the MMP cleavage site GPQQ~IWGQ and a CY3 N-terminus modification (GenScript). 1  $\mu$ M of CY5-conjugated, MMP-cleavable peptide dissolved in cell media was incubated in a plate reader overnight at 37°C with 0.2-1000 ng/mL activated MMP-1, 9, and 13. FRET donor and acceptor fluorescence (530 nm excitation/570 & 670 nm emission) was measured every 5 minutes. Unquenched peptide and MMP-free wells were used as control. 10  $\mu$ L of mineral oil was added to wells to prevent evaporation overnight.

**RESULTS:** The dendron monomer units, MAEA and AXIA thiolamine were successfully synthesized and used to generate a G1 dendron. The yields of the monomer synthesis steps were high (>85%) except for the reaction of the acyl chloride with N-Boc-ethylenediamine, which results in an approximate 1:2:1 ratio of unreacted acyl chloride, desired product with one acyl chloride reacted, and byproduct with both free acyl chlorides consumed. However, the unreacted acyl chloride molecule was recovered for subsequent synthesis. Measurements of GPQQ~IWGQ peptide MMP cleavage indicate slow kinetics with only 3.9%, 5.0%, and 8.0% of Cy3 fluorescence recovered after 16 hours of incubation with 1000 ng/mL MMP-1, 9, and 13 respectively.

**DISCUSSION.** The monomer and dendron synthesis were successful and further optimization is ongoing to obtain ample materials for study. We also successfully prepared a two-color fluorescent contrast agent to measure MMP activity. The measured cleavage of GPQQ~IWGQ containing peptide sequence is slow in the presence of MMP, and, thus, we are exploring alternative peptide sequences that undergo faster kinetics.

**SIGNIFICANCE/CLINICAL RELEVANCE:** This work introduces the design of a novel dual-energy CT contrast agent that will enable early detection and visualization of OA pathophysiology mediated via MMPs, allowing direct clinical evaluation of the efficacy of disease modifying therapies.

**REFERENCES:** [1] A. So, S. Nicolaou, *Korean J Radiol.* **22**, 86–96 (2021). [2] X. Ma et al., *J. Am. Chem. Soc.* **131**, 14795–14803 (2009). [3] R. C. Stewart et al., *J. Med. Chem.* **60**, 5543–5555 (2017). [4] M. P. Lutolf et al., *Proc. Natl. Acad. Sci. U.S.A.* **100**, 5413–5418 (2003).



**Figure 1:** (a) Synthesis of 2-[(methacryloyl)oxy]ethyl acrylate (MAEA) monomer. (b) Synthesis of 2,4,6-trihalo-5-aminoisophthalic acid (AXIA) thiolamine. X indicates iodine (red) and bromine (blue). (c) Scheme for producing iodinated and brominated dendron through sequential addition of MAEA and AXIA thiolamine monomer. **Figure 2:** Dual-energy CT contrast agent with iodinated and brominated dendron linked by MMP-cleavable peptide. Terminal CBP retains iodinated dendron in cartilage matrix while the brominated dendron is free to diffuse allowing relative attenuation to measure MMP activity.