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Effect of Contrast Agent Charge on Visualization of Articular Cartilage Using Computed Tomography: Exploiting Electrostatic Interactions for Improved Sensitivity

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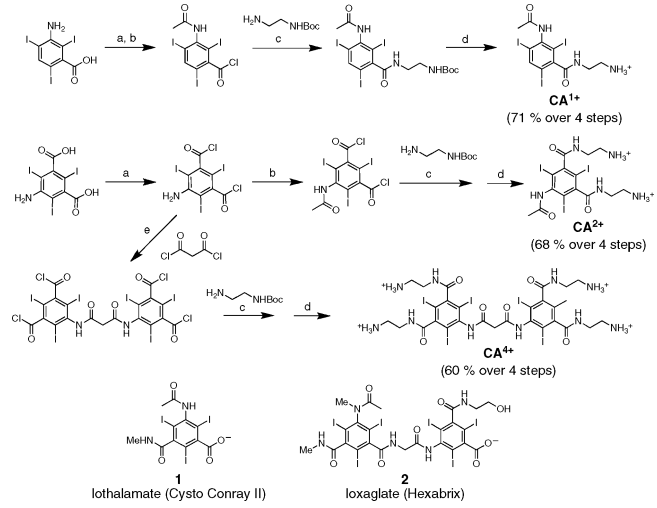
Noncovalent interactions play significant roles in a myriad of biological systems, including molecular recognition in nucleic acids, stabilization of protein structures, and hemodynamical properties of glycocalyx. These various noncovalent interactions (e.g., H-bonding, π-stacking) have been used extensively to synthesize supramolecular structures and as components of drug design using molecular modeling.[1] Application of these principles to the design of tissue-specific medical imaging agents is also of significant interest. For example, contrast agents labeled with targeting antibodies and peptides have been explored.[2] Our focus is on exploiting Coulombic interactions between ionic contrast agents and highly charged polysaccharides,[3] such as those found in cartilage. Here, we report the synthesis of new iodinated cationic computed tomography (CT) contrast agents, their use for visualizing the spatial distributions of glycosaminoglycans (GAGs) in articular cartilage (AC), and preliminary studies exploring the effects of molecular charge on imaging efficacy.

AC is the smooth, hydrated tissue that lines the ends of long bones in load bearing joints. The heavily sulfated and carboxylated polysaccharides comprising the GAGs represent 5−10% by wt of AC and are key components in conferring cartilage with its resistance to compressive loads. The remaining mass of cartilage is composed mostly of collagen (10−20%) and water (68−85%).[4] It has been widely recognized that the loss of GAGs from the AC is a hallmark of osteoarthritis, a degenerative joint disease in which wear or trauma results in damage to the AC surface.[5] Consequently, contrast agents capable of assessing local variations in GAG content are of significant interest for the study of cartilage biology and the diagnosis of cartilage diseases.

Due to the need for quantitative biochemical analysis of tissue samples and cartilage health in patients, both magnetic resonance (MR) and CT imaging modalities have been used to image cartilage *ex vivo* and *in vivo*. To assess GAG content, both imaging modalities use anionic contrast agents.[6] Although these techniques represent the current state of the art, they rely on the limited diffusion of the anionic contrast agents into the target tissue. The contrast agents distribute into the cartilage in inverse proportion to GAG in AC due to the electrostatic repulsion between the contrast agent and the negative fixed charge density of GAGs. We hypothesized that cationic contrast agents would be electrostatically attracted to anionic GAGs and would consequently result in a more sensitive technique for imaging cartilage. Furthermore, our interest lies with CT imaging because it is more widely accessible and less expensive, can image cartilage and bone simultaneously, enables rapid 3D reconstruction of the tissue, and is able to achieve higher spatial resolution over shorter acquisition times compared to MRI. To investigate the effects of contrast agent charge on CT imaging efficiency, we synthesized three new iodinated contrast agent molecules: one having a single positive charge and three iodine atoms (**CA**$^{1+}$), a second having two positive charges and three iodine atoms (**CA**$^{2+}$), and a third having four positive charges and six iodine atoms (**CA**$^{4+}$). These molecules were compared to two commercial formulations of anionic contrast agents, Cysto Conray II (**1**; iothalamate) and Hexabrix (**2**; ioxaglate). These two contrast agent molecules contain 3 and 6 iodine atoms, respectively, but both bear a single negative charge. Cysto Conray II was compared to **CA**$^{1+}$ and **CA**$^{2+}$, and Hexabrix was compared to **CA**$^{4+}$, to minimize differences in molecular structure and enable a direct comparison between charge and imaging sensitivity.

Scheme 1. Synthesis of the +1 (**CA**$^{1+}$), +2 (**CA**$^{2+}$), and +4 (**CA**$^{4+}$) Cationic Contrast Agents[a]



[a] Reagents and conditions: (a) $SOCl_2$, Δ; (b) AcCl, DMA; (c) DMA, DIPEA; (d) CH_2Cl_2/TFA; (e) THF, Δ. Structures of anionic contrast agents **1** and **2** are also shown.

The three cationic iodinated X-ray contrast agents were each synthesized in four steps from commercially available triodinated precursors (Scheme 1). For each contrast agent, the appropriate acyl chloride was generated using published protocols, then reacted with a mono-Boc protected ethylenediamine, and subsequently deprotected with TFA to afford the desired primary amino compounds. The final contrast agents were soluble at concentrations up to 0.2 M in aqueous solutions and bore positive charges, by virtue of their primary amine substituents, at pH $\leq$ 7. The solubility of **CA**$^{1+}$, but not **CA**$^{2+}$ and **CA**$^{4+}$, decreases at higher pH values (7.4); therefore the following experiments were conducted at pH 7.0 so that all the contrast agents could be compared without any solubility problems.

To evaluate the ability of the contrast agents to image AC, we imaged an intact rabbit femur using microcomputed tomography

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(microCT40, Scanco Biomedical, Switzerland). In these *ex vivo* experiments, [I], pH, and osmolality were kept constant across all of the contrast agent solutions to determine the effects of molecular structure and charge on imaging. In each experiment, the distal end of a single rabbit femur was immersed in one contrast agent, imaged using μCT, immersed in saline solution for 24 h to remove the contrast agent, imaged to confirm contrast removal, then exposed to a second contrast agent, imaged, and subjected to histological analysis. This procedure is different from that envisioned *in vivo* where the contrast agent would be delivered via an intra-articular injection.

Images obtained from the rabbit femur studies show that the cationic contrast agents afforded higher X-ray attenuation values and more specific imaging for the cartilage tissue as compared to the anionic contrast agents (Figure 1c). At the low contrast agent concentrations used in this study (15 mg of I/mL of solution, versus 160−300 mg of I/mL for a typical CT arthrography procedure), the anionic contrast agents were largely excluded from the cartilage ECM, resulting in lower attenuation for the tissue. By comparison, the cationic contrast agents achieved higher equilibrium concentrations in the tissue, allowing for facile differentiation between bone, cartilage, and air. The cationic contrast agents had 1.6 (**CA**$^{1+}$), 2.4 (**CA**$^{2+}$), and 2.9 (**CA**$^{4+}$) times higher mean attenuation values for cartilage than their anionic counterparts, indicating that increasing cationic charge yielded higher affinities for the anionic fixed charge density of GAGs.

AC is distinctly stratified in its organization, with the superficial layer being comprised mostly of highly oriented collagen fibrils, while the middle and deep zones have higher GAG content. In addition to increasing the overall attenuation for cartilage, the distribution of the cationic contrast agents also reflects the inhomogeneous distribution of GAGs in each sample. As we hypothesized, the equilibrium distribution of the cationic contrast agents appears to be dominated by electrostatic attraction, such that contrast agent concentration varies proportionately with GAG content.[7] Images obtained after immersion in cationic contrast agents (Figure 1c) had lower attenuation values closer to the cartilage/air interface (superficial zone) and higher attenuation values closer to the cartilage/bone interface (middle and deep zones). This trend is the opposite of what is seen in published data obtained with anionic contrast agents, whose distributions show an inverse relationship with GAG content.[8] The images obtained with **1** and **2** in this study failed to show this trend, primarily due to the lower concentration of contrast agent used. A sample histological section obtained from one of the femurs used in this study shows the natural distribution of GAG for the femoral groove (Figure 1b). A reconstruction of a rabbit femur imaged with **CA**$^{4+}$ highlights the

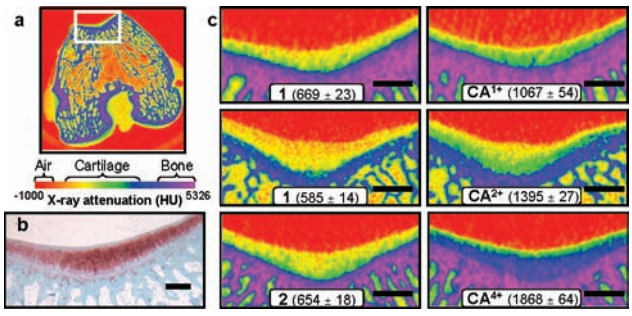

Figure 1. Comparison of anionic and cationic contrast agents. (a) Transverse image of *ex vivo* rabbit femur. Zoomed-in images in (c) highlight the bone−cartilage−air interfaces at the femoral groove (white box). (b) Histological section of femur in **2** vs **CA**$^{4+}$ comparison stained with GAG-specific Safranin-O dye (red color). Scale bar = 1 mm. (c) Representative images from a pairwise comparison of contrast agents, each in a single femur sample (mean cartilage attenuation in Hounsfield Units ± SD). All pairwise comparisons (**1** vs **CA**$^{1+}$; **1** vs **CA**$^{2+}$; **2** vs **CA**$^{4+}$) were statistically significant ($p < 0.0001$) using a student's *t* test comparison. Scale bar = 1 mm.

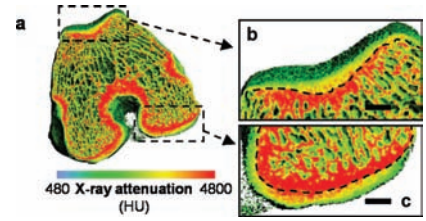

Figure 2. 3D reconstruction of femur after exposure to **CA**$^{4+}$. (a) 60 slices of distal femur, reconstructed. Zoomed-in images of the femoral groove (b) and the medial condyle (c) show that the cartilage can easily be segmented from the bone (dashed line) and that the distribution of the cationic contrast agent reflects the normal distribution of GAG in AC (low GAG in the superficial zone and high GAG in the middle and deep zones). Scale bar = 1 mm.

ability of cationic contrast agents and CT to facilitate monitoring of changes in cartilage attenuation, thickness, and morphology in three dimensions as well as the trabecular architecture of the underlying bone (Figure 2), a task that is difficult to accomplish with MRI.

Taken together, the data presented here represent a compelling case for the continued development of cationic CT contrast agents. Our ongoing experiments are directed toward demonstrating that these cationic contrast agents are able to convey quantitative information about the biochemical characteristics of AC. The suitability of these contrast agents for *in vivo* applications remains to be determined, and issues such as toxicity, administration method, and radiation dose will be the focus of future studies. However, their ability to characterize *ex vivo* cartilage samples is clearly evident. Currently, obtaining data about the spatial distribution of biochemical components in tissue samples is largely accomplished using histology, which is destructive and time-consuming, and thus the use of these contrast agents in conjunction with CT imaging will result in a readily available, nondestructive alternative to histology. We anticipate that the ability to acquire quantitative information about cartilage thickness, morphology, and localized GAG content will aid in the diagnosis and treatment of osteoarthritis as well as the evaluation of new disease modifying osteoarthritis drugs and tissue engineered therapies.

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Supporting Information Available: Full ref 5, detailed synthetic procedures, preparation of contrast agents, imaging protocol, and statistical analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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