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Magnetic Capture of a Molecular Biomarker from Synovial Fluid in a Rat Model of Knee Osteoarthritis

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Abstract

Biomarker development for osteoarthritis (OA) often begins in rodent models, but can be limited by an inability to aspirate synovial fluid from a rodent stifle (similar to the human knee). To address this limitation, we have developed a magnetic nanoparticle-based technology to collect biomarkers from a rodent stifle, termed magnetic capture. Using a common OA biomarker - the c-terminus telopeptide of type II collagen (CTXII) - magnetic capture was optimized *in vitro* using bovine synovial fluid and then tested in a rat model of knee OA. Anti-CTXII antibodies were conjugated to the surface of superparamagnetic iron oxide-containing polymeric particles. Using these anti-CTXII particles, magnetic capture was able to estimate the level of CTXII in 25 μ L aliquots of bovine synovial fluid; and under controlled conditions, this estimate was unaffected by synovial fluid viscosity. Following *in vitro* testing, anti-CTXII particles were tested in a rat

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Author Contributions

EGY and YS conducted the experiments and generated the data sets. Following each experiment, the team (EGY, YS, DPA, JD, and KDA) analyzed the data together, troubleshooted the technology, and designed experiments to develop the concept for magnetic capture of CTXII. KDA and EGY drafted the manuscript and figure, with the team assisting in editing. All authors have approved the final version of the manuscript and figures.

Disclosures

Conflict of Interest

The authors have no conflicts to report

monoiodoacetate model of knee OA. CTXII could be magnetically captured from a rodent stifle without the need to aspirate fluid and showed 10 fold changes in CTXII levels from OA-affected joints relative to contralateral control joints. Combined, these data demonstrate the ability and sensitivity of magnetic capture for *post-mortem* analysis of OA biomarkers in the rat.

Keywords

Magnetic Nanoparticles; Biomarkers; Synovial Fluid; Osteoarthritis; Rodent Model

Introduction

Osteoarthritis (OA) is typically diagnosed through patient reports of pain and disability and verified by changes in joint structure on radiographic scans. While pharmacologic and non-pharmacologic strategies have some ability to improve patient quality-of-life, current OA treatments are primarily palliative³¹. Disease-modifying and preventative strategies may be more successful if OA can be diagnosed earlier in the disease process¹⁰. Toward this end, several research initiatives have emphasized the development of early diagnostic tools for OA^{2,16}. Unfortunately, radiographic evidence of early-stage OA is subtle, and imaging techniques that allow for more sensitive analysis of cartilage loss, such as magnetic resonance imaging (MRI), are cost-prohibitive for OA screening. As such, molecular biomarkers have drawn interest as potential OA diagnostics, and detection of OA biomarkers in urine and serum has shown promise for diagnosing OA prior to MRI, ultrasound, or radiographic scans^{8,11,21}. Since OA is regulated by local catabolic and pro-inflammatory mediators⁷, the earliest molecular evidence of OA will likely be contained in synovial fluid^{3,25}. Recent studies in horses confirm OA biomarker analysis in synovial fluid may have greater potential for early OA diagnosis than analysis of the same biomarkers in serum and urine^{4,17,27}. As with MRI, synovial fluid analysis may be impractical for OA screening, but substantial opportunity exists to use synovial fluid analysis to monitor the effects of traumatic joint injury and to assess OA severity in preclinical studies.

Evaluation of synovial fluid biomarkers in preclinical models, however, has significant challenges. Synovial fluid aspiration from small joints is non-trivial and technologically challenging due to a complex geometry and limited synovial fluid volume. For the rodent stifle (similar to the human knee), synovial fluid aspiration is practically impossible, and as such, biomarker analysis in rodent models is typically conducted *post-mortem* using joint lavage (washing saline through the joint) or fluid wicking (pressing filter paper against the joint surface). Lavage is currently the most widely used technique to investigate joint-level biomarkers in rodent OA models. However, lavage fluids dilute biomarker and introduce errors if fluids are not well-mixed^{1,12}. For fluid wicking, proteins must also be eluted from filter paper, again resulting in biomarker dilution. As such, biomarker analysis following lavage and fluid wicking typically reflect biomarker concentration in the recovered sample; however, a secondary analyte, such as urea, or a known concentration of an internal standard within the lavage fluid can be used to estimate the dilution factor and thereby biomarker concentrations within the joint^{13,23}. However, biomarker concentrations in the joint are susceptible to joint effusion, and measures such as total biomarker in the joint or the rates of

production of a biomarker are likely preferable for OA assessment²⁶. Thus, new technologies to collect OA biomarkers from small synovial fluid volumes may improve our ability to assess OA pathogenesis in small joints.

Over the past decade, the use of magnetic nanoparticle technologies has increased dramatically in a variety of biomedical applications¹⁹. Magnetic nanoparticles are already in clinical use as MRI contrast agents and in biomedical laboratories for cell separation, drug and gene delivery, and remote activation of cell signaling pathways^{5,15,20}. In this study, a new magnetic nanoparticle-based technology (termed magnetic capture) is described for the determination of an OA biomarker in small volumes of synovial fluid *in vitro* and from a rat model of knee OA. Due to the wide use of the c-terminus telopeptide of type II collagen (CTXII)¹⁸ in OA research and the commercial availability of a highly specific, purified, HRP-labeled anti-CTXII monoclonal antibody, CTXII was selected for the development of magnetic capture. Using the CTXII antibody, we demonstrate the ability to assess CTXII in synovial fluid using magnetic capture. In so doing, the stages of magnetic capture are characterized and validated, and the ability of magnetic capture to accurately determine CTXII levels is demonstrated *in vitro* for small volumes of bovine synovial fluid and *post-mortem* in a rat monoiodoacetate (MIA) model of knee OA.

Materials and Methods

Magnetic Capture Concept

Magnetic capture functions through the properties of superparamagnetic iron oxide nanoparticles (SPIONs) embedded within a polymer. In the absence of an external magnetic field, SPIONs do not retain stable magnetization; however in a high-gradient magnetic field, SPIONs experience a translational force directed toward the field source¹⁹. Magnetic capture of OA biomarkers takes advantage of these unique properties: By allowing particles to mix with synovial fluid during incubation and aggregate on a magnetic probe during collection, a molecular target can be isolated from a biological fluid *in situ*.

The conceptual framework for magnetic capture is described in Figure 1. A targeting molecule, such as an antibody, receptor, or aptamer, is conjugated to polymeric particles that contain SPIONs within the particle core. These functionalized magnetic particles are injected into a biological fluid, and following an incubation period, a magnetic probe is inserted into the fluid to collect a portion of the particle-biomarker conjugate. The probe is then removed and particles are released by washing a diamagnetic supernatant across the probe. Non-covalent bonds between the biomarker and targeting molecule are then disrupted using heat, change in pH, or enzymatic cleavage. Magnetic particles are then separated from the collected biomarker using a permanent magnet, and the quantity of particles and biomarkers collected by the probe are quantified through biochemical techniques.

Multiple biomarker quantification methods are theoretically possible following magnetic capture; however, estimation of total biomarker in a fluid can be markedly simplified under the following conditions: 1) Antibody concentrations are much larger than biomarker concentrations, 2) Antibody-biomarker binding is sufficiently strong, and 3) Particle clearance during incubation is negligible (closed system). With these conditions met, initial

biomarker quantity (B_{initial}) can be estimated by multiplying biomarker collected per particle by the quantity of particles injected:

$$B_{\text{initial}} = (\text{Biomarker per Particle}) * \text{Total Quantity of Particles Injected} \quad (1)$$

For *post-mortem* biomarker analysis, the conditions for Equation 1 are easily met, providing a simplified avenue to quantify biomarkers for magnetic capture.

To be clear, other methods of estimating biomarker loads are theoretically possible with magnetic capture, including estimations based upon antibody-biomarker binding kinetics. These methods may be advantageous for *in vivo* applications of magnetic capture. However, biomarker analysis in rodent OA models is typically conducted *post-mortem*; as such, the focus of this work is the validation of magnetic capture through *in vitro* experiments and the demonstration of magnetic capture as a *post-mortem* biomarker assessment method for rodent OA models.

Anti-CTXII Particles

For all experiments described herein, antibodies against CTXII were conjugated to magnetic particles (hereafter referred to as anti-CTXII particles), as follows: Using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) cross-linker, anti-CTXII antibodies were conjugated to carboxylic groups on the surface of polystyrene particles (780 nm diameter) containing fluorescent dye and SPIONs (8.5 nm diameter) within the polymeric matrix (Corpuscular, Inc, Cold Spring, NY, USA). Briefly, 1 mL of magnetic particles (1 % w/v) were washed twice with MES buffer (0.1 M MES, 0.9 % sodium chloride, pH 4.7), then resuspended in 1 mL of MES containing 2 mM EDC and 5 mM Sulfo-N-hydroxysulfosuccinimide (NHS) for 15 min at room temperature (RT). Then, 20 mM of 2-mercaptoethanol was added to quench the EDC. Particles were briefly washed with PBS (137 mM NaCl, 2.7 mM KCl, 12 mM Phosphate Buffer, pH 7.4, containing 2 mM EDTA, all cross-linking materials obtained from Thermo Scientific, Rockford, IL) and mixed with 100 μL of peroxidase conjugated anti-CTXII antibody (ImmunoDiagnostic Systems, Copenhagen, Denmark). Particles were reacted with anti-CTXII antibodies for 5 h at RT, transferred to 4°C for 16 h, then dissolved in PBS containing 2 mM EDTA and 10 mM of hydroxylamine to hydrolyze unreacted NHS. After 10 min in hydroxylamine, particles were washed three times with PBS containing 0.05% TWEEN-20, 2% BSA, and 2 mM EDTA (hereafter referred to as Buffer 1), then dissolved in Buffer 1 to a final volume of 1 mL. The amount of antibody attached to the particles was determined by mixing anti-CTXII particles (5 μL , 300 $\times$ dilution) with ultra-3,3',5,5'-tetramethylbenzidine (50 μL , TMB) peroxidase substrate, reacting for 15 min, then stopping the reaction by addition 0.18 M sulfuric acid (50 μL). Optical density at 450 and 650 nm was then measured on a Synergy 2 Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT) with calibration curves created from antibody stock solution (Cartilaps ELISA kit).

Magnetic Particle Quantification

For particle quantification, magnetic particles were suspended in 5 μL of Buffer 1 in a 0.2 mL PCR tube, then read for fluorescence intensity at 530/590 nm using Synergy 2 Multi-

Mode Microplate Reader. Solutions of known particle concentrations were used as standards.

CTXII ELISA

CTXII levels were determined using Serum Preclinical Cartilaps ELISA, according to the manufacturer's specifications. Briefly, diluted biotinylated anti-CTXII antibody was incubated in streptavidin-coated microwells for 30 min. Microwells were washed with buffer provided in the kit. Samples were then added, incubated for 1 h, then washed with buffer. A dilution of secondary anti-CTXII peroxidase-conjugated antibody was added to the microwells, incubated for 1 h, and washed with buffer. TMB peroxidase substrate solution was added for 15 min, then stopped by addition of 0.18 M sulfuric acid. Microwell optical density was measured at 450 and 650 nm using the Synergy 2 Multi-Mode Microplate Reader.

Hyaluronidase Treatment of Synovial Fluid

To investigate the effects of synovial fluid viscosity, a portion of bovine synovial fluid (Animal Technologies, Inc., Tyler, TX, USA) was treated with 0.01 mg/mL of hyaluronidase from bovine testes (Type I-S, Sigma-Aldrich Corporation, St. Louis, MO) for 4.5 h at RT. Hyaluronidase-treated and native (untreated) synovial fluid were stored at -20°C and thawed prior to use.

Assessing Heat Release of CTXII

In magnetic capture, biomarkers bind to functionalized magnetic particles via non-covalent bonds (Figure 1B); and once extracted on the magnetic probe (Figure 1C), biomarkers are released from the particle-biomarker complex to quantify the particles and biomarker collected (Figure 1D). To assess the ability of heat to release CTXII from an anti-CTXII antibody, anti-CTXII particles were mixed with 90 μL of bovine synovial fluid such that final antibody concentrations of 0, 55, 110, 275, 550, 1350, or 2700 pM were achieved, incubated for 2 h at RT, then separated from synovial fluid via 10 min of centrifugation at 18,000 g. Particles were re-suspended in 52.5 μL of Buffer 1 and heated to 85°C for 3 min. Following heating, supernatant was isolated from particles via 10 min of centrifugation at 18,000 g. CTXII collected by particles and released by heat, CTXII not collected by particles and remaining in synovial fluid, and CTXII in synovial fluid aliquots not exposed to anti-CTXII particles were quantified via ELISA.

Assessing Dynamic Binding Equilibrium

A dynamic binding equilibrium should be established prior to particle-biomarker collection (Figure 1B), and time to achieve dynamic binding equilibrium will depend on antibody concentration, biomarker concentration, and the rate constants for formation and dissociation of the antibody-biomarker complex. Since synovial fluid is highly viscous, rate constants may depend on the ability of anti-CTXII particles to diffuse through the fluid. To assess the conditions related to Equation 1, 1.5 billion anti-CTXII particles (300 antibody molecules per particle) were suspended in 100 μL of Buffer 1, then added to 400 μL of hyaluronidase-treated or native bovine synovial fluid. After 0.3, 2.5, 5, 10, 20, and 40 min of

incubation, a 50 μL aliquot was removed with magnetic particles isolated via centrifugation at 18,000 g for 10 min. Isolated pellets were suspended in 50 μL of Buffer 1, heated to 85°C for 3 min, then analyzed using the CTXII ELISA.

Assessing the Effects of Fluid Viscosity on Particle Collection

After magnetic capture, the amount of particles collected must be sufficiently large for both particle and biomarker quantification. This collection may depend on the particles' ability to move through synovial fluid. To assess this concern, 1.9 billion anti-CTXII particles (460 antibody molecules per particle) in 30 μL of Buffer 1 were mixed with 300 μL of either hyaluronidase-treated or native bovine synovial fluid, incubated with gentle mixing for 1 h, then divided into 25 μL samples of approximately 150 million particles per sample. A cylindrically shaped NdFeB magnet (1 mm diameter by 1 mm length, Grade N50, Super Magnet Man, Birmingham, AL) was then inserted in native synovial fluid samples for 0.5, 1, 2, 4, 8, 15, 30, 60, or 120 min or in hyaluronidase-treated synovial fluid for 0.5, 1, 2, 4, 8, 15, 30, 60, or 120 s, then removed. Particles were removed from the magnet by repeatedly washing the magnet surfaces with 200 μL of Buffer 1 ejected from a 20 μL micropipette tip. A strong magnetic plate placed beneath the well prevented re-attachment of particles to the cylindrical magnet (Nanotherics Magnefect Nano II, Staffordshire, United Kingdom). Buffer used for washing was removed from the well, and particles were suspended in a known volume of Buffer 1 (40 μL). Following particle collection, CTXII was released from collected particles using 3 min of 85°C heat. Particles collected and particles remaining in synovial fluid were quantified via fluorescence. CTXII concentrations were assessed using a CTXII ELISA.

In Vitro Validation of Magnetic Capture in Multiple Bovine Synovial Fluid Samples

To demonstrate magnetic capture *in vitro*, anti-CTXII particles were mixed with 25 μL of hyaluronidase-treated or native bovine synovial fluid. Following 1 h of incubation, NdFeB magnets were inserted in synovial fluid for 2 min. Particles were then washed from the magnet using 200 μL of Buffer 1 in the presence of a strong magnetic plate with supernatant removed after washing. Collected particles were re-suspended in 40 μL of Buffer 1, with 2.5 μL aliquoted for particle concentration and 37.5 μL heated to 85°C for 3 min and assessed for CTXII as described above.

Post-mortem Magnetic Capture of CTXII from the Rat Stifle

All studies described herein were conducted under IACUC-approved protocols at the University of Florida. Moreover, all injections of magnetic particles occurred immediately after humane euthanasia (cardiac puncture and exsanguination under deep anesthesia); blood sera samples were acquired from whole blood acquired during exsanguination using blood-separating vacutainers (BD, Franklin Lakes, NJ), according to the manufacture's instructions.

To assess the feasibility of magnetic capture in a rat stifle, six Sprague-Dawley rats (90 day old, male) received an intra-articular injection of 180, 360, or 720 million anti-CTXII particles ($n=4$ stifles per group). In addition, eight Sprague-Dawley rats received an intra-articular injection of MIA (3 mg in 25 μL of saline) at 90 day old; injections were conducted

with a 29 ½ gauge, U-100 insulin syringe, and OA was allowed to develop for 30 days after injection. An additional 8 animals were used as age-matched naïve controls for the MIA animals (120 day old). For the MIA-injected animals and the respective naïve controls, 720 million anti-CTXII particles were injected into the stifle joints. Anti-CTXII particles were allowed to incubate for 2 h prior to magnetic collection. For magnetic collection, a 16 gauge catheter was inserted into the femoral groove, followed by the insertion of a cylindrical magnet through the catheter and into the joint space (1 mm diameter by 1 mm length N50 NdFeB magnet attached to a steel rod, Super Magnet Man, Birmingham, AL). Particles were collected on the magnet for 10 min, then the magnet was removed and washed to isolate particles. Collected particles were heated to 85°C for 3 min, followed by quantification of collected particles and CTXII.

Following magnetic collection, joint tissues were processed for histology using standard paraffin-embedding practices. At least one 10µm thick frontal sections was acquired for every 100 µm from the anterior horn of the medial meniscus to the posterior horn of the meniscus. Sections were stained with toluidine blue, with the section that represented the most severe degeneration on the tibial plateau selected for OARSI histopathology grading²².

Statistical Analysis

Differences between groups were assessed using 1-way analysis of variance and a post-hoc Tukey's HSD test, when indicated (Statistica, Tulsa, OK).

Results

Heat Release of CTXII

Figure 2 shows CTXII collected by anti-CTXII particles and released by heat (triangles, dotted line), CTXII not collected on the anti-CTXII particles and remaining in the synovial fluid (squares, solid line), and CTXII in synovial fluid samples not exposed to anti-CTXII particles (dash-dot-dash line). As expected, higher concentrations of CTXII antibody resulted in higher amounts of CTXII bound to anti-CTXII particles and in lower amounts of CTXII remaining in the synovial fluid. Heating samples to 85°C for 3 min was able to break non-covalent bonds between the antibody and CTXII, and the sum of CTXII collected and CTXII remaining in the fluid compared well to CTXII levels in synovial fluid aliquots not exposed to anti-CTXII particles. Combined, these data demonstrate heat can recover CTXII from anti-CTXII particles after magnetic collection.

Dynamic Binding Equilibrium

Time to dynamic binding equilibrium is a function of both antibody and biomarker concentration. With anti-CTXII antibody concentrations 14 fold higher than the initial CTXII concentration, 97% of the CTXII was bound by anti-CTXII particles in both hyaluronidase-treated and native synovial fluid after 20 min, and 99% of CTXII was bound by the anti-CTXII particles after 40 min. These data demonstrate a dynamic equilibrium is easily established with 1 h incubations when antibody concentrations exceed biomarker amounts by 14 fold or more, and that time to binding equilibrium is not appreciably altered by synovial fluid viscosity for the above conditions.

Effects of Fluid Viscosity on Magnetic Particle Collection

Collection of magnetic particles on an NdFeB magnet was strongly influenced by synovial fluid viscosity (Figure 3), where magnetic collection was much faster in hyaluronidase-treated synovial fluid relative to native synovial fluid (compare white and black shapes). However, the percentage of particles collected compared favorably with the percentage of CTXII collected at each collection time for native synovial fluid (Figure 4A); thus, the CTXII per particle was relatively independent of synovial fluid viscosity. As such, the amount of CTXII in the sample predicted by Equation 1 was relatively consistent for each collection time (Figure 4B); and as long as sufficient material was collected for biomarker and particle quantification, CTXII levels calculated from Equation 1 compared favorably to the initial amount of CTXII in the synovial fluid (2.4 pg , $111 \pm 4 \text{ pg/mL}$).

In Vitro Validation of Magnetic Capture in Multiple Bovine Synovial Fluid Samples

CTXII amounts predicted via magnetic capture were not statistically different from CTXII amounts in aspirated fluid samples in 3 different native synovial fluid samples and 1 hyaluronidase-treated sample (Figure 5); hence, under the proper conditions, CTXII amounts in synovial fluid can be approximated using magnetic capture *in vitro*. The variability of magnetic capture is larger than a direct ELISA on a synovial fluid aspirate. This is expected as magnetic capture requires additional processing steps that will increase the variability of the measure; however, to be very clear, a direct ELISA is not an option for rodent OA models due to the technological and practical limitations of synovial fluid aspiration in rats and mice.

Magnetic Capture of CTXII from the Rat Stifle

Detectable levels of CTXII were recovered from all twelve 90 day old naïve rat stifles. While CTXII per particle was dependent on the number of particles injected (Figure 6A), CTXII within the joint, as predicted by Equation 1, was not dependent upon the number of particles injected (Figure 6B). Thus, as long as the particles are not saturated with biomarker, magnetic capture can be used to estimate biomarker levels within an articular joint. Magnetic capture was also able to assay the amount of CTXII in a rat model of knee OA, where elevated levels of CTXII were detected in OA-affected joints relative to the contralateral stifle and naïve controls (Figure 6C). Assessments of CTXII in the serum of these same animals did not yield statistically significant differences between MIA-injected and age-matched naïve control animals (Figure 6D), despite significant evidence of joint destruction in MIA-injected stifles (Figure 7). These data demonstrate the utility of biomarker analysis within the OA-affected stifle and the ability of magnetic capture to facilitate these analyses *post-mortem*.

Discussion

These data demonstrate the ability to estimate CTXII amounts in a biological fluid using magnetic capture. The critical variable for magnetic capture is biomarker per particle, where it is essential the particles are not saturated with biomarker. Under these conditions, the ratio of biomarker per particle can be used to estimate total biomarker within a joint. While some variation exists between biomarker levels predicted by magnetic capture relative to levels

assessed in an aspirated fluid sample, this variability is minimal relative to the expected differences in biomarker levels between OA-affected and control joints. Moreover, since magnetic capture does not require fluid to be aspirated, magnetic capture can be useful for small articular joints where synovial fluid aspiration is challenging, such as the rodent stifle.

As with any biochemical assay, researchers should be informed on the limitations of their assay. Magnetic capture will have an upper limit associated with particle saturation and a lower limit based on the biochemical assay. Figure 2 demonstrates how the saturation limit can be determined: When the amount of added antibody is smaller than that of the biomarker (0 to 300 pm), the amount of collected biomarker increases as antibody concentration increases, indicating anti-CTXII particles cannot bind more CTXII. As the number of anti-CTXII antibodies continues to increase, plateaus occur in both CTXII collected and CTXII remaining. For these conditions, free antibody still exists on the particle surface, indicating particles are not saturated. Importantly, if particles are not saturated, CTXII in the rat stifle was not dependent on the amount of particles injected into the joint (See Figure 6).

Using magnetic capture, total CTXII within the MIA-injected joints was 10 fold higher than contralateral and age-matched naïve stifles ($p < 0.001$). Comparable work has been conducted in the rat MIA model using lavage, where only 2 fold changes in CTXII concentration were observed between MIA-injected joints and saline controls¹⁸. Moreover, lavage assessment of CTXII showed significantly larger variability, with additional animals needed to show differences between groups ($n = 19-20$).

It should be noted we are using magnetic capture to measure total biomarker in the joint, not biomarker concentration. Since total biomarker in the joint space is not affected by joint effusion, it may be preferred to concentration for many biomarkers. However, assuming an estimated synovial fluid volume of 10–100 μL for the rat stifle, the CTXII concentration would be approximately 150–1500 pg/mL in the naïve stifle and 1000–10,000 pg/mL in the MIA-injected stifle. As a reference, CTXII concentrations were approximately 200 pg/mL in the synovial fluid from healthy horses and 400 pg/mL in a horse osteochondral chip model of OA⁴. Given the aggressive, whole-joint destruction seen in the rat MIA model relative to the focal cartilage defects seen in the horse osteochondral chip model, estimated changes in CTXII concentrations detected by magnetic capture are reasonable relative to synovial fluid CTXII concentrations reported for the horse.

It should also be noted most studies utilizing lavage report biomarker concentrations in the lavage fluid, not necessarily concentration of biomarker in the joint. Since the initial synovial fluid volume is unknown, a secondary analyte is necessary to estimate intra-articular concentrations of a biomarker following lavage¹². Conversely, magnetic capture follows a precipitation-recovery principle that involves a self-calibration to the amount of particles injected (See Figure 1F). Since the number of particles injected is known, the amount of biomarker in the joint can be calculated assuming the particles are dispersed though the joint, particle clearance from the joint is negligible (closed system), and particles are not saturated. This calculation of biomarker amount is independent of the initial volume of the sample; however, because the volume is not known, concentration cannot be calculated using this application of magnetic capture.

Finally, lavage fluid also dilutes biomarkers, often below the level of detection, and necessitates fluids to be well-mixed prior to extraction, a requirement that is difficult to achieve and confirm. Conversely, the level of detection for magnetic capture is primarily defined by the detection limits for the biochemical assay (the CTXII ELISA in this study) and the efficiency of magnetic collection. The detection limits for the biochemical assay are independent of magnetic capture, and several methods could be used to boost the collection efficiency for biomarkers found in low concentrations, such as increasing collection time or increasing the magnetic susceptibility of the particles. In general, the greater the amount of magnetic and biological material collected during the collection phase (Figure 1C), the higher the likelihood of biomarker detection for magnetic capture. Moreover, with high collection efficiencies, the magnetically-driven concentration of biomarkers may actually allow for biomarker detection in samples that were originally at concentrations below the detection limits.

To be clear, researchers using lavage assume losses are relatively equivalent in all samples, and thus, the differential level of biomarker between a diseased joint and an internal control is informative of the disease process. We agree these data are informative; however, a method that provides better control and measurement of the sources of variability should be more sensitive and repeatable. Magnetic capture achieves this by 1) measuring total biomarker rather than concentration measures that are sensitive to effusion, 2) self-calibration to the amount of particles injected, allowing losses to be standardized between samples, and 3) by magnetically-precipitating the biomarker target within the joint space, rather than diluting the target with a lavage fluid. Combined, these effects may explain the differences between our results and prior work using lavage, and indicate magnetic capture may have improved sensitivity for the *post-mortem* assessment of intra-articular CTXII levels relative to the commonly used technique of joint lavage.

This proof-of-principal work was conducted in the rat MIA model of knee OA; however, preclinical OA research is more commonly conducted in the mouse due to the availability of transgenic models. As noted above, MIA injection is a relatively aggressive model of OA compared to many surgical models of post-traumatic OA. Nonetheless, magnetic capture of CTXII should be scalable to other models as long as the amount of biomarker collected from the OA-affected stifle is above the detection limits of the ELISA. This constraint will likely result in further refinement of the magnetic capture procedure, including increased particle collection times, but does not necessarily prevent the use of magnetic capture in other rodent models of knee OA.

Ultimately, OA diagnosis may require the assessment of multiple biomarkers or the comparison of biomarker ratios within a joint. The purpose of this study is to demonstrate the utility of magnetic capture for biomarker analysis, and thus, the detection of a single biomarker was advantageous as an initial step. The distinguishing advantage of magnetic capture is *in situ* isolation of a biomarker from a biological fluid, whereas the method of quantifying the biomarker target does not actually vary from that of an aspirated fluid. In this paper, ELISA was used to quantify CTXII levels both after magnetic collection and in bovine synovial fluid aspirates. Thus, by creating magnetic particles that target other

biomarkers or by attaching multiple targeting molecules to the magnetic particles, magnetic capture could be adapted for multiplex analysis of biomarkers in the future.

Biomarker collection was done *post-mortem* in this study, and *post-mortem* biomarker assessment is typical for rodent OA models. However, magnetic capture could theoretically be adapted for *in vivo* use, and we hope to develop the technology for these purposes in the coming years. For *in vivo* collection, the assumption of a closed system will need to be closely evaluated, as small particles and proteins can clear rapidly from the joint space and this clearance rate can be affected by the disease state³⁰. Biosafety concerns of the targeting molecules and particles will need to be properly addressed for this application, but it should be noted SPION associated technologies are FDA-approved as contrast agents for magnetic resonance imaging¹⁴. In addition, this work used SPIONs embedded in polystyrene. However, magnetic capture implies no limitations on the polymeric matrix material, and polystyrene could be replaced in the future with more biocompatible polymers, such as dextran or polyglycolic acid. Significant concerns and challenges will need to be further explored to advance this technology to *in vivo* use, but the work described herein demonstrates the utility of magnetic capture as a *post-mortem* laboratory assessment tool for OA biomarkers in rodent models.

This work describes magnetic capture under the condition where antibody concentration is much greater than the concentration of the targeted biomarker. Since many biomarkers are found in picomolar concentrations, this condition does not necessarily mean the concentration of antibody will be prohibitively large. In fact, nanomolar ranges of antibody will generally be able to satisfy this condition for most current OA biomarkers^{3,28}. Alternate methods of assessing biomarker levels following magnetic capture are theoretically possible. As an example, when only trace amounts of antibody are needed such that the concentration of the antibody is far below that of biomarker, the initial concentration of the biomarker could be estimated through the law of mass action, provided the dissociation constant for antibody-biomarker binding falls between healthy and OA levels for the biomarker.

Interestingly, the magnetic collection of particles was three orders of magnitude slower in native synovial fluid relative to hyaluronidase-treated synovial fluid. While synovial fluid viscosity was not directly assessed (samples were exhausted for other experiments), the apparent change in magnetic capture time approximately matches the expected difference in viscosity between native (~100–400 centipoise) and hyaluronidase-treated (~1 centipoise) synovial fluid: 2–3 orders of magnitude^{9,24,29}. Since the time for a magnetic particle to move over a given distance is predicted to be proportional to the viscosity of a fluid⁶, the quantity of magnetic material collected in a given time may be able to roughly approximate synovial fluid viscosity or at least changes in the synovial fluid mechanics. Of course, since synovial fluid is a non-Newtonian fluid, this measure would not be as detailed or as accurate as rheological assessments of fluid viscosity. However in small fluid volumes, magnetic capture may be able to indirectly assess synovial fluid mechanics without the need to aspirate the fluid, which may provide useful diagnostic information in and of itself.

This work demonstrates CTXII can be concentrated and removed from small volumes of synovial fluid using anti-CTXII particles and a magnetic collection device. With controlled

conditions, CTXII collected via magnetic capture can estimate CTXII amounts in the original sample, both *in vitro* and *post mortem* in a rat OA model. This work lays the foundation for the future development of *in vivo* magnetic capture, where parameters such as particle clearance and biosafety must be further considered. Nonetheless, *post-mortem* magnetic capture of OA biomarkers may support the assessment and development of biomarkers in small animal models by enabling the extraction of biomarker data from small rodent joints where fluid aspiration is technically challenging.

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Figure 1. Conceptual framework for magnetic capture of an OA biomarker

First, a targeting molecule for an OA biomarker (antibody, receptor, or aptamer) is conjugated to the surface of a polymeric particle that contains superparamagnetic iron oxide nanoparticles within the polymeric matrix, creating a functionalized magnetic particle. Functionalized magnetic particles are then injected into the joint (A) and allowed to bind the targeted OA biomarker until a dynamic equilibrium is reached (B). A permanent magnet is then inserted into the synovial fluid, and a portion of the magnetic particle-biomarker complex is removed from the joint space on the magnet (C). The permanent magnet is processed to remove magnetic particles (D). Biomarkers are then separated from the functionalized particles by breaking the bond between the biomarker and targeting molecule (E). Particles and biomarkers are quantified (F), then related to the initial concentration or total biomarker within the joint.

Figure 2. Heat release of CTXII from anti-CTXII functionalized magnetic particles

Higher amounts of anti-CTXII particles, and thus higher concentrations of anti-CTXII antibody, bound more CTXII biomarker during the 2 h incubation period, resulting in less free CTXII biomarker in the synovial fluid (black squares, solid line). Heating the sample to 85 °C for 3 min resulted in the breakdown of the non-covalent bond between CTXII and the antibody and recovery of the CTXII biomarker from the anti-CTXII particles (black triangles, dotted line). Moreover, the sum of CTXII collected on the magnetic particles and the CTXII remaining in the fluid for synovial fluid (X) was comparable to the initial level of CTXII in the synovial fluid sample (dot-dash-dot). The initial concentration of CTXII in the synovial fluid sample was 111 ± 4 pg/mL.

Figure 3. Magnetic particle collection is influenced by synovial fluid viscosity

The number of particles collected in hyaluronidase-treated (white triangles) and native synovial fluid (black triangles) increased with magnetic collection time, with a corresponding drop in particles remaining in hyaluronidase-treated (white circles) and native synovial fluid (black circles), respectively. The difference in magnetic collection time (2–3 orders of magnitude) is approximately equivalent to the change in fluid viscosity. Native synovial fluid has a viscosity of approximately 100–400 centipoise^{9,29}, whereas hyaluronidase-treated synovial fluid has a viscosity near that of water, 1 centipoise²⁴. Values are presented as mean $\pm$ standard error (n=3).

Figure 4. With sufficient material, magnetic capture can accurately assess the CTXII concentration in a synovial fluid sample

Following particle collection in native synovial fluid (described in Figure 3), CTXII was released from collected particles using heat and CTXII concentrations were assessed using a CTXII ELISA. CTXII collected (white squares) and particles collected (black squares) increased with particle collection time (Panel A) and compare favorably with each other at most collection times. Using the ratio of CTXII to particles, the amount of CTXII in the synovial fluid was predicted for each collection time (Panel B). Collection times of 1 min and above yielded predictions comparable to the initial amount (2.4 pg CTXII). The initial

concentration of CTXII in the synovial fluid ample was 97 ± 3 pg/mL. Values are presented as mean $\pm$ standard error (n=3).

Figure 5. CTXII amounts assessed via magnetic capture compare favorably to CTXII amounts as assessed via ELISA

CTXII amounts and concentrations predicted via magnetic capture were reasonably similar to CTXII concentrations measured by ELISA in all samples. The initial CTXII concentrations were 101 ± 7 (Native Sample 1), 137 ± 4 pg/mL (Native Sample 2), 260 ± 5 pg/mL (Native Sample 3), and 156 ± 3 pg/mL (Hyaluronidase-Treated Sample). Coefficients of variation (CV) are reported for each group, and the variability of magnetic capture is larger than a direct ELISA on a synovial fluid aspirate. This result is expected as an ELISA occurs in both procedures, but magnetic capture requires additional processing steps. The additional processing should increase the variability; however, to be very clear, a direct ELISA is not an option for rodent OA models (see Figure 6) due to the technological and practical limitations of synovial fluid aspiration in rats. Values are presented as mean $\pm$ standard deviation ($n=3$).

Figure 6. Intra-articular CTXII levels can be assessed in a rat model of knee OA using magnetic capture

Anti-CTXII particles were injected into left and right stifle joints of 90 day old naïve rats (180, 360, or 720 million particles, $n=4$ per group), then collected with a permanent magnet. CTXII per collected particle is shown in Panel A; the saturation limit for the particles was 418 CTXII sites per particle. Using equation 1, the predicted amount of biomarker in the joint space was similar regardless of the amount of particles injected into the joint (Panel B), demonstrating that, under proper conditions, magnetic capture can yield a stable prediction of biomarker load in a rat joint. Magnetic capture was also used to assay the amount of CTXII in a rat model of knee OA. Elevated levels of CTXII were detected in OA-affected joints, significantly higher than both contralateral stifle and naïve controls (Panel C). Moreover, assessments of CTXII in the serum of these same animals did not yield statistically significant difference between MIA-affected and naïve animals (Panel D). These data demonstrate the utility of biomarker analysis within the OA-affected stifle and the ability of magnetic capture to facilitate these analyses.

Figure 7. Histological evidence of joint destruction following intra-articular of MIA in the rat stifle

Representative histological images are shown for contralateral limb controls and MIA-injected stifle joints (Panel A); images represent the medial joint space. Significant degeneration was seen in the MIA group, while no degeneration was found in contralateral limb controls (Panel B). Values are presented as mean $\pm$ standard deviation.