

Development of the Interferon-Gamma (IFN- γ) Biomarker for the Use
of Magnetic Capture in Osteoarthritic Rat Models

Jorge Figueras

Supervisory Committee:

Dr. Kyle Allen

Dr. Blanka Sharma

Dr. Jon Dobson

Oral Defense

April 17, 2018

Abstract:

Early detection of osteoarthritis (OA) is critical to slow the progression of this maladaptive joint disease. Molecular biomarkers in the joint (synovial fluid) may indicate the state and severity of OA. Our laboratory developed a magnetic capture technology proven to be more effective than prior techniques for biomarker collection from animal joints (1,2). Interferon-gamma (IFN- γ) is currently being investigated and developed as a potential biomarker for magnetic capture technology. The capture and quantification of IFN- γ will lead to better characterization of OA's disease model.

The development of a biomarker for the magnetic capture technology involves adjustment of conditions for the biomarker collection and particle handling. These conditions include load of IFN- γ antibodies on particles, number of particles needed to capture biomarker from an animal knee, and time needed for antibody to bind to biomarker. To find these conditions, the antibody affinity to biomarker must be determined and the correct antibody must be selected with a suitable affinity.

The experiments conducted thus far include: determining levels of IFN- γ in rat serum, maximum load of antibodies on particles, antibody affinity to biomarker, and optimal pH for assaying the biomarker. We determined the maximum load of IFN- γ antibodies on particle, antibody affinity to the biomarker, and the post-release conditions for the IFN- γ biomarker. Before moving towards *in vivo* conditions, the time needed for the antibody to bind to the biomarker and the best pH for biomarker release must also be determined.

Introduction:

Osteoarthritis(OA) is currently diagnosed through patient reports of pain in the knee along with further verification through several diagnostic tests such as joint fluid aspiration with subsequent test for biomarkers, radiography imaging, and magnetic resonance imaging(MRI). However, when trying to evaluate the progression of OA in a small animal model, various constraints arise due to the nature of the anatomical differences. More specifically, the very small volume of synovial fluid of rodents, $\sim 1 \mu\text{L}$ in mice, and $\sim 40 \mu\text{L}$ in rats, makes direct joint aspiration technically impossible (3). Along with joint aspiration not being feasible to evaluate early stages of OA in rodent models, the small anatomical features of rodent knees make it difficult to use clinical imaging modalities. These constraints have made it difficult for the evaluation of novel pharmaceutical therapies for the treatment of OA in rodent models.

Our laboratory developed a magnetic capture technology proven to be more effective than prior techniques for biomarker collection from animal joints (1,2). The conceptual framework for the magnetic capture technology is shown in Figure 1. Magnetic capture employs superparamagnetic nanoparticles embedded within a polymer. These particles do not retain stable magnetization in the absence of magnetic field, but acquire strong magnetization when the field is present. Antibodies specific to the biomarker of interest are conjugated to the polymer surface. These complex particles are injected into the synovial fluid of rodents. After a certain amount of time in

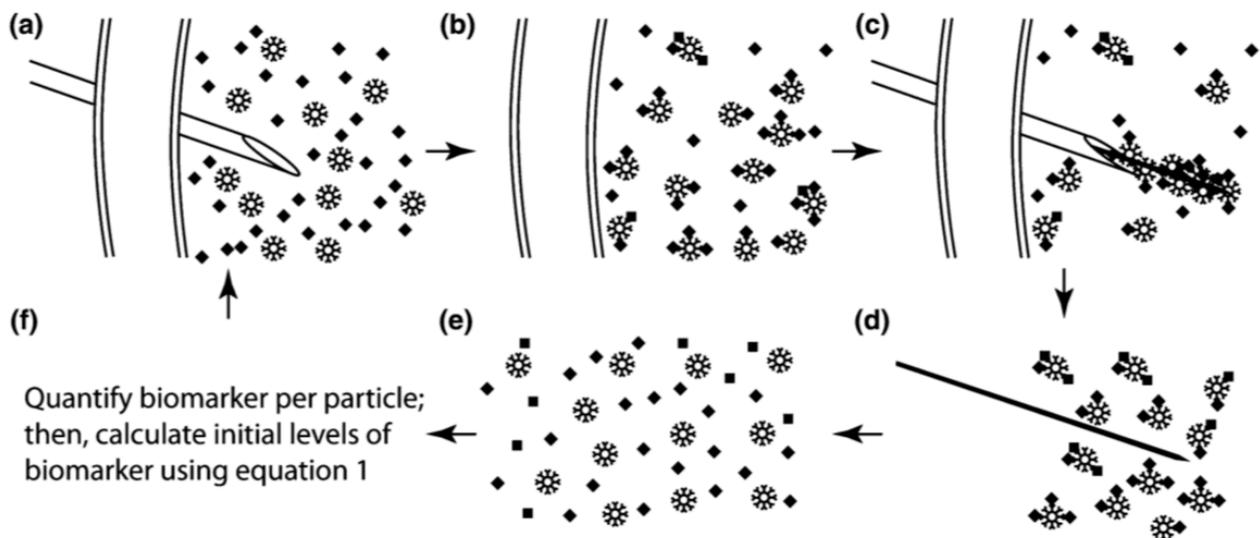


Figure 1 Conceptual framework for magnetic capture of an OA biomarker (1).

the synovial fluid, the particles are then extracted out of the knee space using an external magnetic field and the biomarkers that have been collected are then counted.

Interferons (IFNs) are ubiquitous cytokines produced by the immune system. They trigger antiviral as well as antiproliferative responses to target cells. There are three classes of IFNs: I, II, and III. IFN type I and III are associated with innate immunity, whereas IFN type II is associated with adaptive immunity. IFN- γ is a type II interferon associated with viral, bacterial, and parasitic infections (4). Since IFN- γ is associated with bacterial infections, it can serve as indicator of septic inflammation that may occur during animal surgery during the process of inducing OA. Thus, IFN- γ can have potential for being a biomarker of interest for other applications outside of the OA field. For example, the magnetic capture technology for the IFN- γ biomarker can be beneficial in specific tissues that require targeted diagnostic tools, which the magnetic capture technology can offer.

Prior to conducting any in-vitro experiments with the IFN- γ biomarker and the magnetic capture technology, proper conditions for the biomarker collection and particle handling must be identified. These conditions include appropriate particles, right load of the antibody on particles, number of particles needed to capture biomarker from an animal knee, and time needed for antibody to bind to biomarker. To find these conditions, one needs to determine the antibody affinity to biomarker and use an antibody with suitable affinity. One also needs to determine conditions for biomarker release from particles (ex. optimal pH), a biomarker detection method (ex. ELISA).

Molecule biochemistry: IFN- γ has been characterized as a homo-dimeric glycoprotein that depends equally on both of its subunits for proper physiological function during immunogenic responses. The IFN- γ receptor complex consists of two chains: IFN- γ R1 (the major ligand-binding subunit) and IFN- γ R2 (necessary for IFN- γ signal transduction and increases affinity of IFN- γ R1 for its ligand) (5). The IFN- γ 's isoelectric point, a protein's pH when the net charge is zero, is 8.72 (6). Understanding the isoelectric point of IFN- γ is important because of its potential role in determining the biomarker's optimal pH for future experiments.

Molecule pathophysiology in OA: IFN- γ is produced by activated natural killer (NK) cells, CD4⁺ helper-1 (Th1) cells, CD8⁺ T cytotoxic cells, $\gamma\delta$ T cells, natural killer T (NKT) cells, macrophages, dendritic cells, naïve CD4⁺ T cells, and B cells (7). T lymphocytes are responsible for most IFN- γ production in the body. IFN- γ R1 level in patients with OA are decreased, which gives rise to the possibility for an upregulation of IFN- γ in the synovial fluid due to the decrease in the uptake of the molecule. The literature, however, does not show any experimental data that proves or disproves this hypothesis.

Biomarker Levels in Serum and SF: Literature shows a healthy Wistar rat has a basal IFN- γ level of 220.83 pg/mL (5). Synovial fluid IFN- γ levels in rodents have not been reported. The lack of literature containing synovial fluid IFN- γ levels in rodents may be due to the lack of technology that can quantify such low quantities of concentration.

Detection Methods for Biomarker: The detection method chosen for quantification of IFN- γ was the V-PLEX Rat IFN- γ Kit (Meso Scale Diagnostics (MSD), Cat. #K153QOD). The V-PLEX platform offers a cost-effective, validated immunoassay for IFN- γ that promises the highest sensitivity (0.65 pg/mL) on the market. Another advantage of MSD assays is the opportunity to utilize multi-spot assays that can detect several biomarkers in one sample simultaneously. For future directions with the magnetic capture technology, this will be extremely beneficial for the assaying of an array of biomarkers of interest to understand the progression of OA from various parameters.

Capture Antibody: The capture antibody chosen for IFN- γ was Biotin anti-rat IFN- γ Antibody from BioLegend®. This specific antibody is polyclonal, which is advantageous for the collection of biomarkers in the synovial fluid. Polyclonal antibodies are relatively inexpensive and easy to produce, compared to monoclonal antibodies. Polyclonal antibodies often have a high affinity (avidity) towards a target protein – a product of their ability to recognize multiple epitopes on a particular antigen. With regards to the capture technology, this is beneficial because it can essentially amplify a signal from a particular target protein from low concentrations. Especially when working with small rodent synovial fluid volumes, this allows for more accurate and sensitive detection methods.

Particles: The particles chosen were Dynabeads™ MyOne™ Streptavidin C1. This particle is considered the gold standard (Thermo Fisher®) for isolation and handling of biontynylated antibodies because the high binding affinity of streptavidin-biotin interactions ($K_d=10^{-15}$) (7). These particles are superparamagnetic, meaning that they exude magnetic properties in the presence of a magnetic field. The particles are coated with streptavidin, which binds to the biontynylated capture antibodies for IFN- γ .

Materials and Methods:

V-PLEX Rat IFN- γ Kit: The V-PLEX assay is a sandwich immunoassay, as shown in Figure 2. Each well is pre-coated with capture antibodies that are specific to the analyte of interest, which is IFN- γ in this case. Samples containing the analyte are loaded into each well and incubated for a given amount of time. This incubation period is then followed by the addition of a detection antibody tagged with electrochemiluminescent labels into each well. The assay utilizes electrochemiluminescence (ECL) to emit light as voltage is applied to the sample. The intensity of measured light is proportional to the amount of analyte in the sample. This method is ideal for samples with wide range of concentrations because the applied voltage can be scaled to amplify

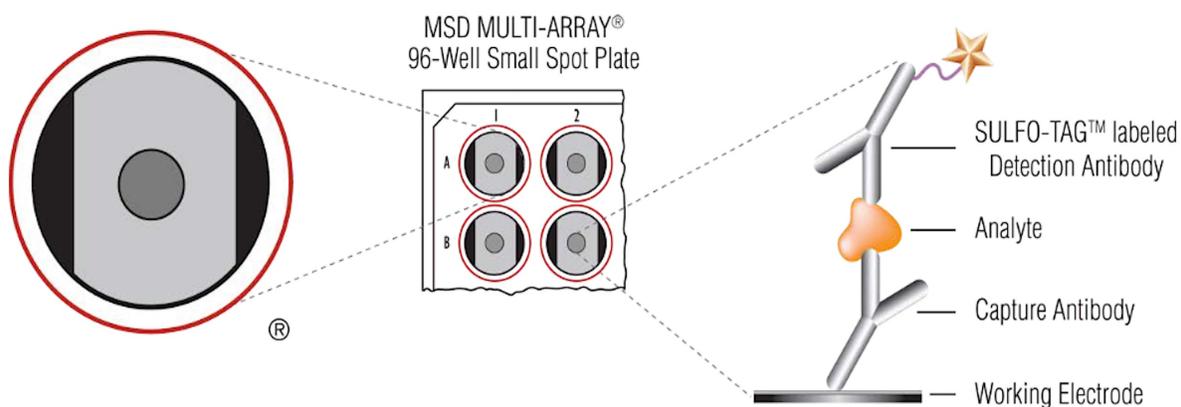


Figure 2 Small Spot plate diagram showing placement of analyte capture antibodies. The specific capture antibody is pre-coated on the well, represented as the structure directly connected to the working electrode. The analyte then binds to the capture antibody in the well, which is followed by a SULFO-TAG™ detection antibody. A voltage is applied to the working electrode at the bottom of the figure, which causes light to be emitted through a series of cascade reactions (10).

the signal and detect small, as well as large amounts of analyte. All of the V-PLEX assays conducted followed the protocol provided by MSD and used the MSD reagents.

The general protocol was as follows: addition of 150 μL Blocker H to each well, 1 hour incubation on shaker at room temperature, wash each well with 150 μL of Wash Buffer (PBS + 0.05% Tween-20), addition of 50 μL of prepared samples, 2 hour incubation on shaker at room temperature, wash, addition of 25 μL of detection antibody, 2 hour incubation on shaker at room temperature, wash, addition of 150 μL of Read Buffer T to each well, and then read on the MSD instrument. The signal output is displayed as arbitrary units (AU). For specific experiments, slight modifications were made to the protocol. These variations dealt mostly with the type of buffer used to suspend the IFN- γ in solution.

Analysis: For analysis, ECL signal was plotted versus IFN- γ concentrations. MSD software offers analysis of data from their own instrument, although we preferred our own. Excel was used to analyze the data. More specifically, a linear fit model as well as a four-parameter logistic curve fit for serial dilutions of calibrator was used to calculate the lower limit of detection (LLOD). The Excel add-in program, Solver, was used to curve fit by finding optimal values for four-parameter logistic curve fit. The LLOD was calculated by multiplying a coefficient (2.58) to the standard error of the linear fit's y-intercept to the data divided by the linear fit's slope. The coefficient chosen corresponds to the 99% confidence interval. The coefficient of determination, R^2 value, was also calculated for certain experiments and was an indicator for linearity.

Actual Levels in Serum: IFN- γ levels were assayed from four various rat serum samples from different experiments. The samples were stored at -20°C for various time periods (depending on when the animal experiments were conducted). One particularly interesting serum sample was from a Lewis rat that was sacrificed because of an infection. Each sample underwent a single, four-fold dilution, as recommended by MSD. Concentration were calculated by comparing sample signal measurements to the signal measurement of calibrator samples of known concentrations. A linear-fit model and a four-parameter logistics curve were used to analyze the data.

Particles: Particles were counted in order to determine the amount of biomarker initially in the knee based off the formula:

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

Particle counting must be done as a part of any *in vivo* experiments. For particle counting, absorbance at 450 nm was used. Absorbance was plotted versus known particle concentrations for calibration.

Max Load of Antibodies on Particles: The maximum number of antibodies conjugated onto each particle was determined by the manufacturer (20 ng of Biotin anti-rat IFN- γ Antibody per 1 μg of particles, Dynabeads™ MyOne™). Our laboratory previously tested the manufacturer's estimate with antibodies to other biomarkers and found it true. We currently use this estimate for new antibodies in biomarker magnetic capture experiments.

Antibody Affinity to Biomarker: A three-day experiment was conducted to determine the dissociation constant (K_d). As seen in Figure 3, Biotin anti-rat IFN- γ antibodies were conjugated to the particles on the first day. On the second day, the antibody-particle conjugates were mixed with IFN- γ molecules to capture and release the biomarker. On the third day, released IFN- γ molecules were assayed.

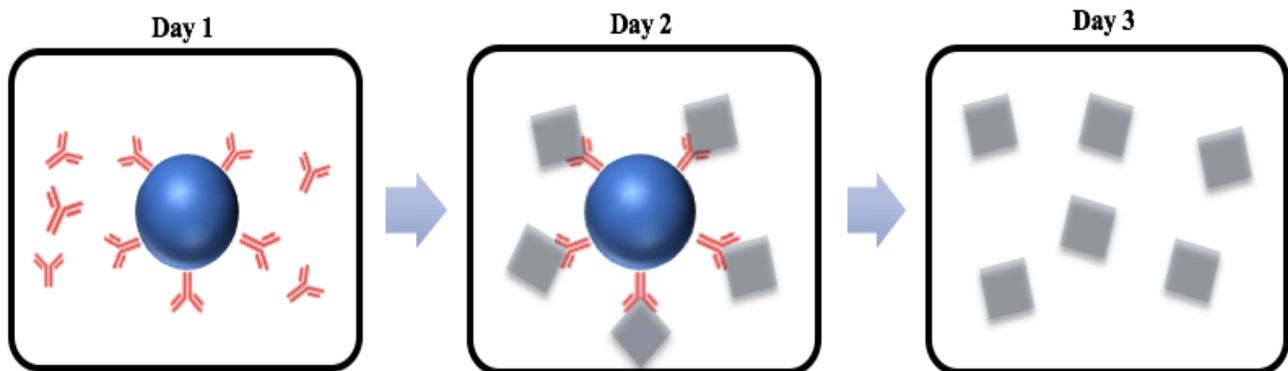


Figure 3: General schematic of the capture and release experiment conducted to measure K_d . Day 1: Target capture antibody was added to the nanomagnetic particle solution. Day 2: Conjugated nanomagnetic particles were incubated with the target biomarker. After the incubation, the particles were released. Day 3: Released biomarkers were assayed.

On day one, the target capture antibody amount (2000 ng per sample) was added to a solution of particles (100 μg per sample), incubated for 2.5 hours on a tube revolver (10 rpm) at room temperature, and then incubated at 4°C overnight. The overnight incubation allows time tight binding of the antibody on the particles, assuming that all of the available streptavidin sites on the particles were taken by capture antibodies. The particles were washed and suspended in Buffer 1 (PBS + 2% BSA + 2 mM EDTA). This buffer was tested and found most suitable for conjugated particles handling.

On day two, two dilutions of a stock IFN- γ aliquot (26,600 pg/mL) were made. One was diluted in Buffer 1 and the other in Buffer 2 (Glycine-Tris buffer, pH 8.3 with 2% BSA and 2 mM EDTA). The IFN- γ /Buffer solution was mixed with the conjugated particles from the previous day and was incubated for 2 hours on the tube revolver. The particles were then placed on a magnetic plate and the supernatants were removed and kept in a separate tube. The pellet of particles was re-suspended in Buffer 2, incubated on the tube revolver for 10 minutes, and placed on a magnetic plate again to remove the supernatant. The supernatant was added to the previous supernatant pool. The supernatant collected was labeled as “Unbound IFN- γ “. The particles were then washed with Buffer 1. For biomarker release from the particles, the Buffer 3 (Glycine-Tris buffer, pH3.1 with 2% BSA and 2 mM EDTA) was added, and the tubes were incubated for 15 minutes on the tube revolver. The particles were placed on a magnetic plate and the supernatant was removed and placed in a tube containing 1M Tris base to neutralize the supernatant and was labeled as “Captured and Released IFN- γ ”. Two calibrator strips were simultaneously prepared on day 2: (1) Calibrator in Buffer 1, (2) pH Treated Calibrator. The pH treated calibrator followed the same procedure the particles went through to capture and release IFN- γ , however, particles were not introduced to the calibrator solutions.

On day three, the four strips were assayed: the calibrator in Buffer 1, the calibrator that was pH treated, the supernatant of the unbound particles, and the supernatant of the bound/released particles. The signal measurements were analyzed using two strips of calibrator samples of known concentrations. A linear-fit model and a four-parameter logistics curve were used to analyze the data.

Optimal Post-Release Condition: After the biomarker is released from the antibody and prior to the start of the MSD assay, an optimal pH of the assay buffer must be determined. To determine optimal pH for the IFN- γ biomarker in the assay, various pH buffers were incubated with the biomarker for one hour at room temperature and then at 4°C over night. As shown on Figure 4, the various pH buffers tested started at pH 1.93 and ended at pH 9.36. A second experiment was conducted focused on pH buffers starting at 7.61 and 9.36. This experiment was performed in duplicate because of results gathered after the initial experiment. All of the pH dependence experiments were conducted with IFN- γ diluted in Tris-Glycine buffer (pH range 1.93 - pH 9.36).

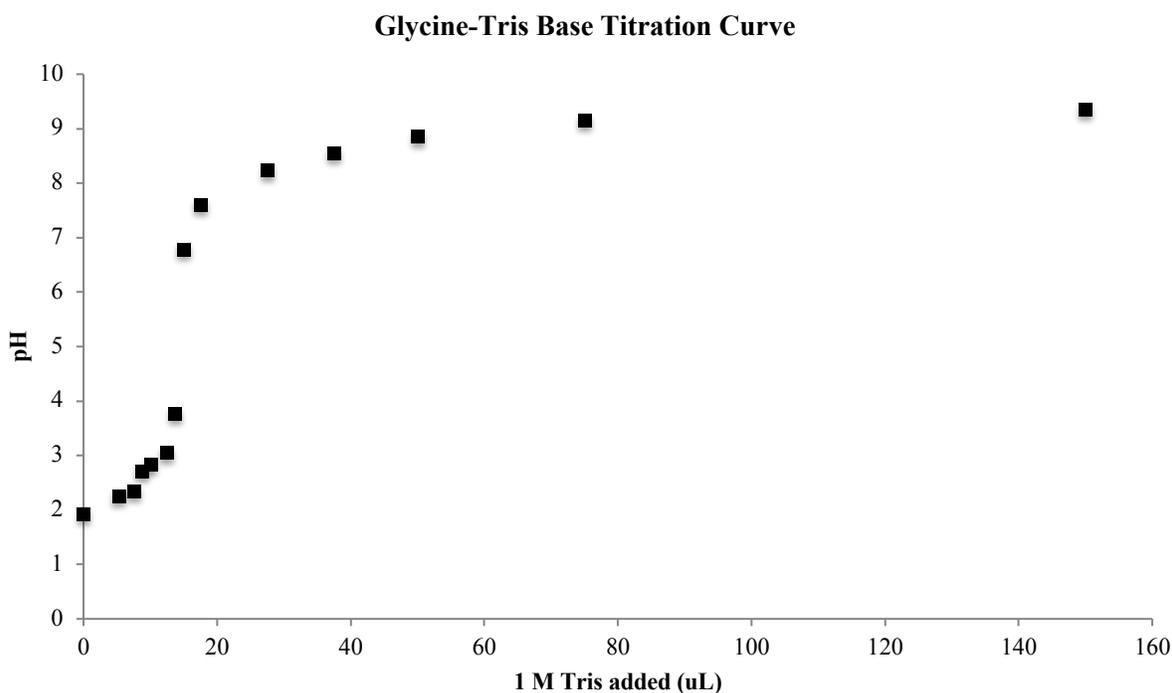


Figure 4: The Glycine-Tris base buffers used to test for the best pH for MSD assay. The buffers tested has pH values of 1.93, 2.18, 2.35, 2.71, 2.75, 3.01, 5.22, 6.71, 7.57, 8.22, 8.48, 8.74, 9.14, and 9.36

Additionally, an experiment was conducted to determine the effects of treating the IFN- γ biomarker with pH conditions similar to those of the release conditions to determine the effects of low pH on the biomarker stability. This pH treatment began with IFN- γ suspended in Buffer 3 (Glycine-Tris buffer, pH3.1 with 2% BSA and 2 mM EDTA) and then was neutralized using 1M Tris Base. This test would determine if the biomarker would be destroyed when applying the pH treatment to release it from the nanoparticle.

Results and Discussion:

Actual Levels in Serum: As seen in Figure 5, IFN- γ levels in rat serum were all lower than the LLOD (1.5 pg/mL). The LLOD was calculated from the two calibrator strips ran simultaneously with the serum samples, and were prepared as recommended by the manufacturer using the required reagents. The LLOD was calculated using a liner fit model and was also verified using a four-parameter logistical fit model on MATLAB.

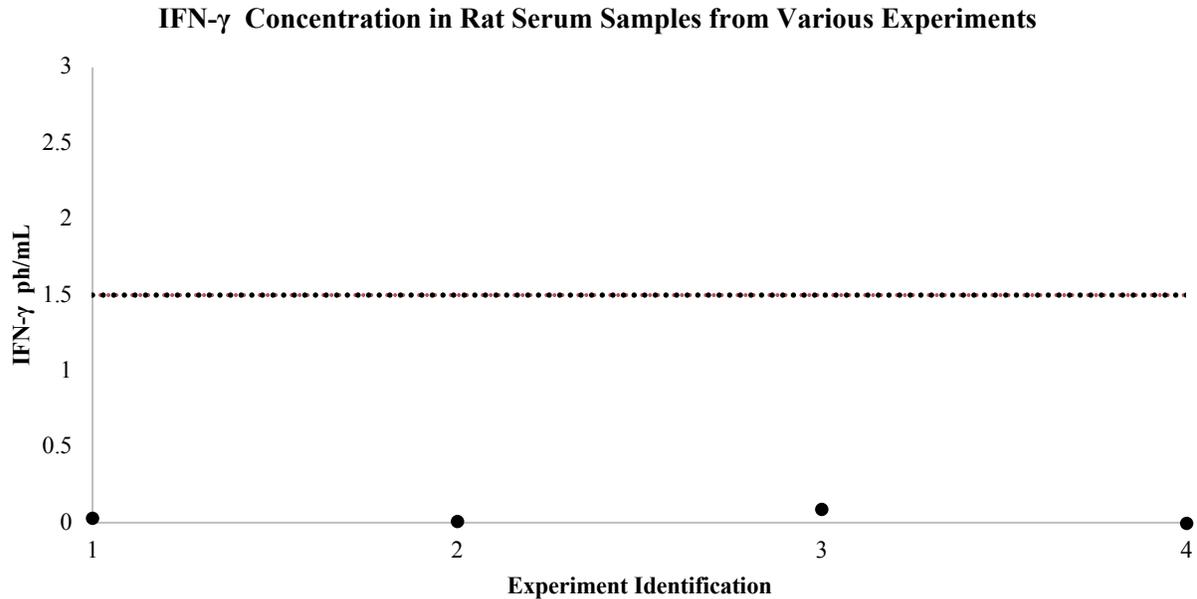


Figure 5: IFN- γ concentrations from various rat experiments. Each experiment is classified by date(s) they were conducted on. (1) 02/17-18/2016 (2) 06/27/2017 (3) 12/08/2016 (4) 12/16/2016.

There was one serum sample (Experiment Identification #2) from a sacrificed rat that had reported infection acquired from animal surgery. Increased cytokine concentration was expected in this serum sample, but various possibilities could have led to the lack of measured signal. The multiple freeze/thaw cycles the serum samples went through could have had an adverse effect on the stability of the IFN- γ biomarker. Additionally, whether the infection was truly systemic or local was not recorded and could possibly be why there was no signal read from that sample.

All of the other rat serum samples (Experiment Identification #1, #3, and #4) had concentration readings below the LLOD.

Particles: The calibration graphs for the particles of known concentrations were all linear, as shown in Figure 6. The linearity and precision from the results validates the results as a calibrator for future experiments to count the number of particles of unknown concentrations.

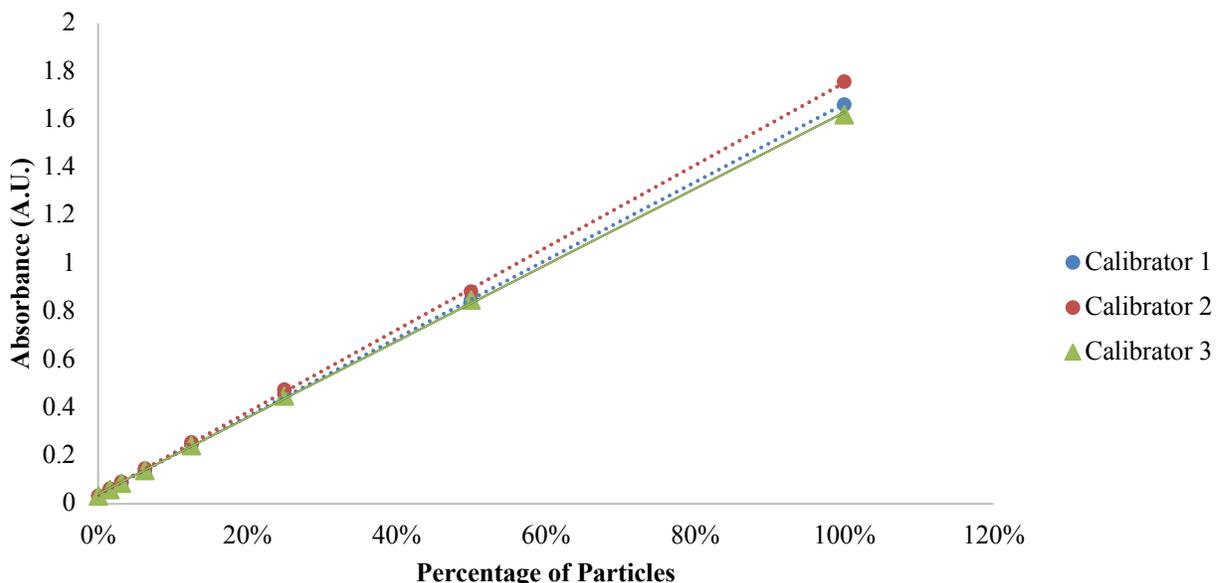


Figure 6: The results from three different solutions that underwent serial dilutions. For particle counting, absorbance at 450 nm was used. Absorbance was plotted versus known particle concentrations for calibration. This data will be used for future experiments to determine the number of particle in a solution of unknown concentration.

Antibody Affinity to Biomarker: As seen in Figure 7, the binding affinity is calculated to be 25 nM. The ascending and descending curves both appear to be mirror images of each other, which is expected and is an indicator of no major experimental error. The sum of the unbound IFN- γ and the captured and released IFN- γ had an average of 91%, which is also expected because the sum should theoretically be 100%. The concentrations of IFN- γ were calculated using a using a four-parameter logistical fit model on Excel. The pH treated calibrator strip was used as the standard to calculate the concentrations for the ascending curve. The estimate for K_d is based on the assumption that the concentration of antibody is substantially higher than the concentration of total biomarker, which was true due to the high concentration of antibody used compared to IFN- γ concentrations.

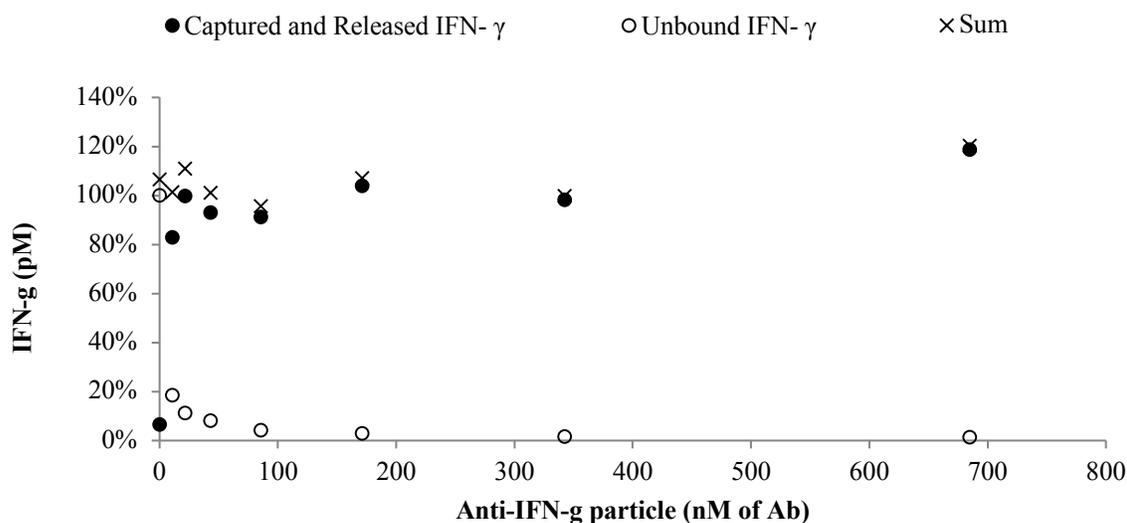


Figure 7: The capture and release curve of the IFN- γ biomarker. This data corresponds to the 3-day experiment conducted, as outlined previously in the Materials and Methods section. The Unbound IFN- γ corresponds to the IFN- γ molecules that were collected from the supernatant of the conjugated particles. The Captured and Released IFN- γ corresponds to the IFN- γ molecules that were bound to the particle and then released using the pH treatment.

Optimal Post-Release Condition: The signals from the pH buffers, as seen in Figure 4, were read. No clear trend could be deduced from the first experiment that tested signals from the pH buffers of a larger range of pH, as seen in Figure 8. This experiment corresponded to the buffers with pH values of 1.93, 2.18, 2.35, 2.71, 2.75, 3.01, 5.22, 6.71, 7.57, 8.22, 8.48, 8.74, 9.14, and 9.36. A second experiment was conducted where the initial IFN- γ was doubled and higher pH buffers were tested in duplicate. The pH buffers used in the second experiment has pH values of 7.57, 8.22, 8.48, 8.74, 9.14, and 9.36. The data collected from the second experiment is shown in Figure 9. There is a scatter in signal towards pH 8 and the reason is unknown. Biomarker stability is currently being tested in the 8.5 to 9 pH regions.

A separate experiment was conducted with the treatments that the IFN- γ undergoes during the process of unbinding from the nanoparticle with the outlined procedure previously mentioned. The results, as seen in Figure 10, show no drastic change compared to the stability of IFN- γ experiences in the standard Buffer 1 solution. The only minor difference is that IFN- γ signal begins to scatter slightly in lower concentrations, which may be due to the low pH treatment effecting the stability of IFN- γ during the treatment.

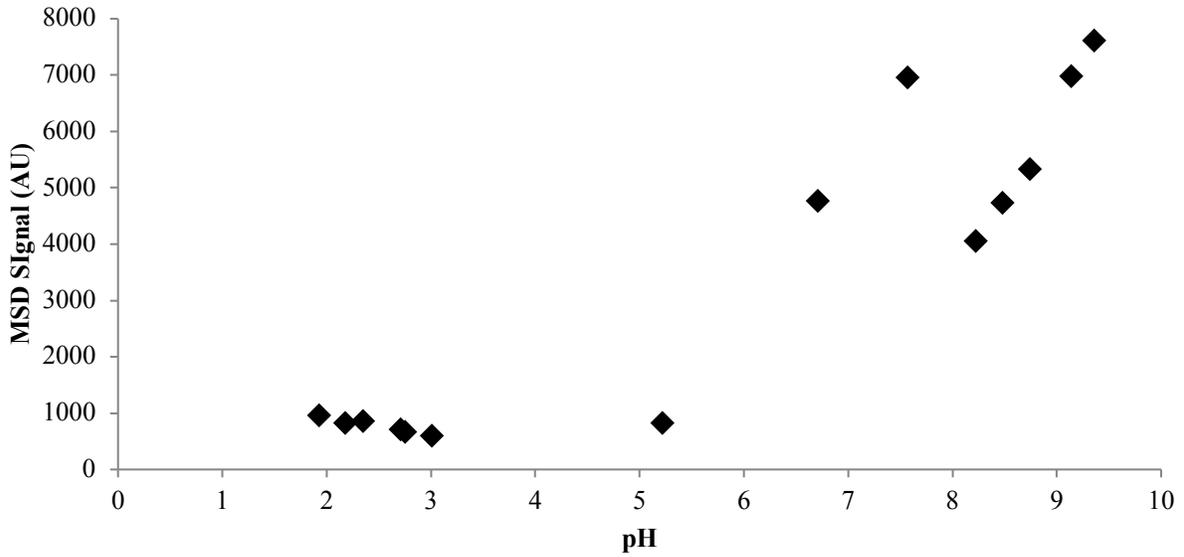


Figure 8: IFN- γ in various pH buffer solutions plotted against their respective MSD reading. This experiment investigated IFN- γ stability in a large range of buffers with pH values of 1.93, 2.18, 2.35, 2.71, 2.75, 3.01, 5.22, 6.71, 7.57, 8.22, 8.48, 8.74, 9.14, and 9.36.

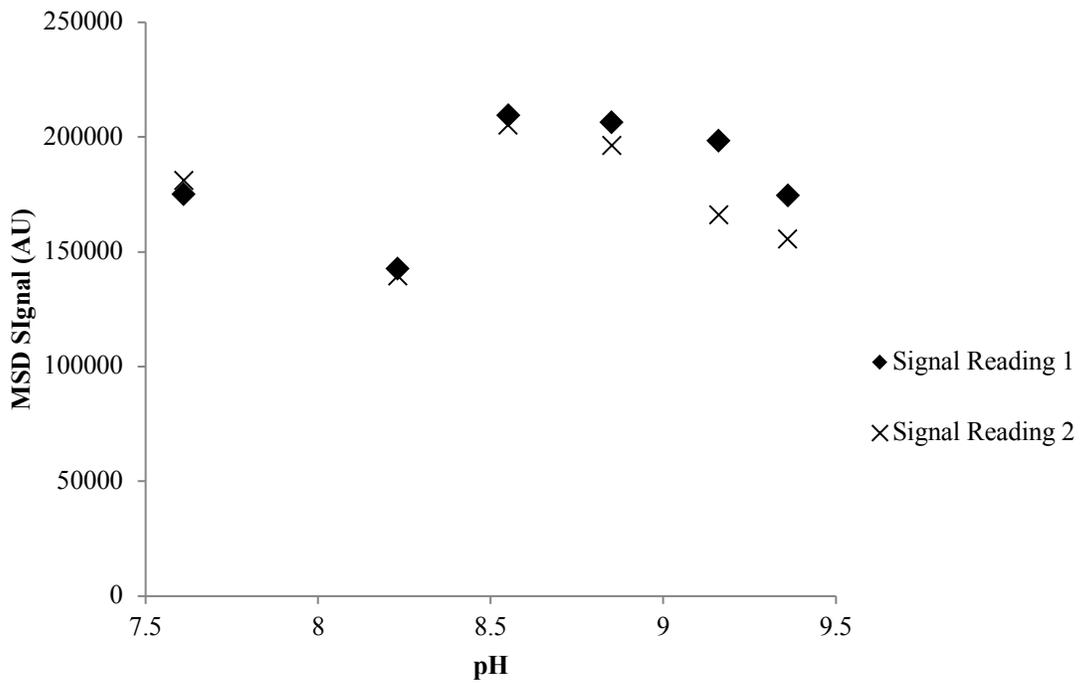


Figure 9: Doubled Concentration of IFN-g with narrower pH Range in Duplicates. The second experiment conducted to determine the optimal pH for biomarker using the MSD platform. The pH buffers used in the second experiment has pH values of 7.57, 8.22, 8.48, 8.74, 9.14, and 9.36.

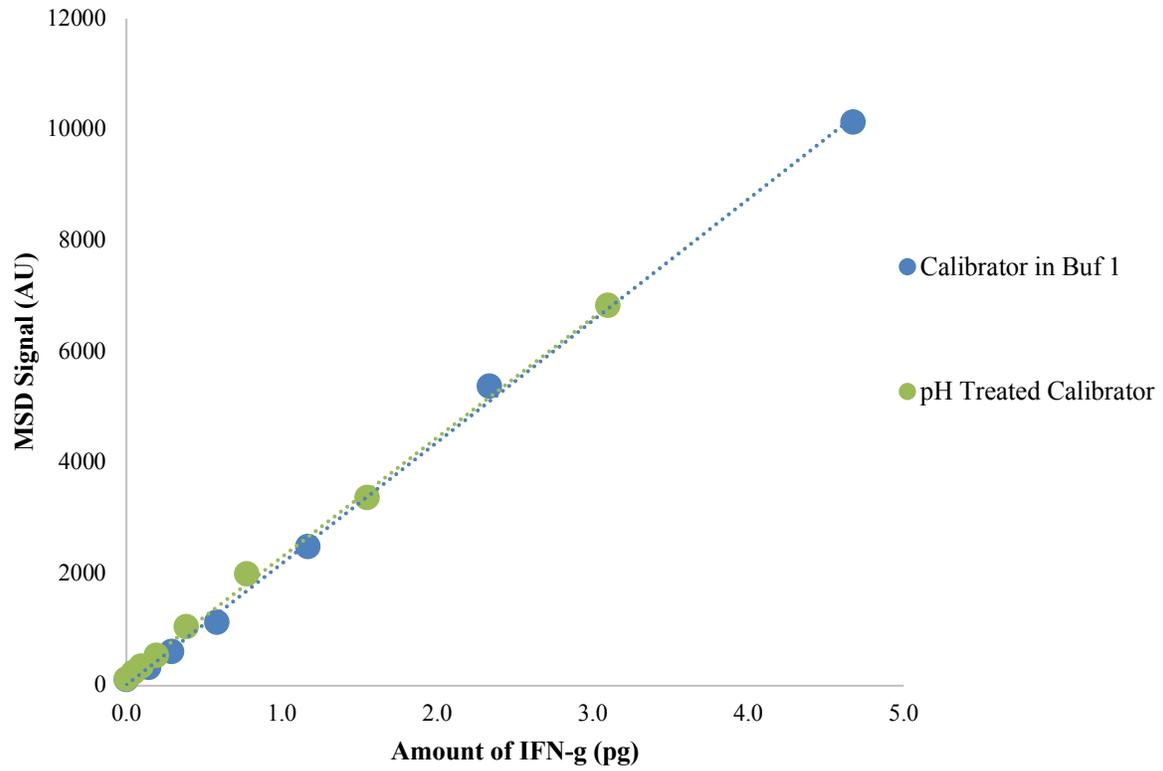


Figure 10: Various concentrations of IFN- γ that have undergone similar treatment during the capture and release of the biomarker from the particle. The calibrator in Buf 1 (Buffer 1) showed similar signal for the calibrator that underwent pH treatment. This shows that the pH treatment does not affect the stability of the biomarker goes under pH treatment

Conclusion:

Preliminary steps have been conducted to magnetically collect IFN- γ *in vitro*. So far, we set up a platform to calculate the number of particles in a given solution of unknown particle concentrations, the binding affinity of the IFN- γ biomarker to its antibody was determined, and we investigated the optimal pH for the biomarker concentration assay. With regards to the particle counting, we were able to obtain a calibration curve that we will use for future *in vivo* experiments to ultimately determine the amount of biomarker in a knee. The binding affinity of the IFN- γ biomarker to its antibody is strong enough to proceed with further experiments. Having a strong binding affinity is ideal because it assures that we will be able to collect almost all of the IFN- γ biomarker when moving towards *in vivo* experiments. Based on this affinity, a minimal amount of particles the needs to be injected in an animal knee can be calculated.

A major constraint observed during experimentation of post-release conditions for the biomarker was the instability of signal observed in the pH range of 7 to 9. This instability is uncertain and further investigation must be done to determine how the instability of the IFN- γ biomarker in this pH range may affect the detection of IFN-during an *in vitro* or *in vivo* experiment.

To avoid the issues of detection of the biomarkers that we have observed, other assaying techniques may be utilized. More specifically, fluorescently-tagged antibodies may be a viable solution to the current detection problem. Fluorescently-tagged antibodies can be used to quantify the amount of biomarker that is attached on a nanoparticle without having to release the biomarker from the nanoparticle. Our lab is currently investigating this option.

Further experiments may be conducted to modify current protocols and conditions. In the future, the time needed for the antibody to bind to the biomarker, the actual magnetic collection from serum, the number of particles needed for an animal knee will all be determined and explore pH conditions for biomarker release.

References:

- [1] Yarmola, Elena G., Yash Shah, David P. Arnold, Jon Dobson, and Kyle D. Allen. "Magnetic Capture of a Molecular Biomarker from Synovial Fluid in a Rat Model of Knee Osteoarthritis." *Annals of Biomedical Engineering* 44.4 (2015): 1159-169. Web.
- [2] Yarmola, E.g., Y.y. Shah, H.e. Kloefkorn, J. Dobson, and K.d. Allen. "Comparing intra-articular CTXII levels assessed via magnetic capture or lavage in a rat knee osteoarthritis model." *Osteoarthritis and Cartilage* 25.7 (2017): 1189-194. Web.
- [3] Seifer DR, Furman BD, Guilak F, Olson SA, Brooks SC, Kraus VB. Novel Synovial Fluid Recovery Method Allows for Quantification of a Marker of Arthritis in Mice. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society.* 2008;16(12):1532-1538. doi:10.1016/j.joca.2008.04.013
- [4] Meyer O. Interferons and autoimmune disorders. *Joint Bone Spine.* 2009;76(5):464-473. doi:10.1016/j.jbspin.2009.03.012.
- [5] Heidarianpour A, Vahidian Rezazadeh M, Zamani A. Effect of Moderate Exercise on Serum Interferon-Gamma and Interleukin-17 Levels in the Morphine Withdrawal Period. *International Journal of High Risk Behaviors & Addiction.* 2016;5(2):e26907. doi:10.5812/ijhrba.26907.
- [6] "Interferon gamma." *Interferon gamma : Protein Overview : UCSD-Nature Molecule Pages.* N.p., n.d. Web. 03 Aug. 2017.
- [7] Teixeira, Leonardo K., Bruna Pf Fonseca, Bianca A. Barboza, and João Pb Viola. "The role of interferon-gamma on immune and allergic responses." *Memórias do Instituto Oswaldo Cruz* 100.Suppl 1 (2005): 137-44. Web.
- [8] Heidarianpour A, Vahidian Rezazadeh M, Zamani A. Effect of Moderate Exercise on Serum Interferon-Gamma and Interleukin-17 Levels in the Morphine Withdrawal Period. *International Journal of High Risk Behaviors & Addiction.* 2016;5(2):e26907. doi:10.5812/ijhrba.26907.

[9] Dynabeads™ MyOne™ Streptavidin C1. Dynabeads MyOne Streptavidin C1 - Thermo Fisher Scientific. <https://www.thermofisher.com/order/catalog/product/65001>. Accessed April 2, 2018.

[10] "V-PLEX Rat IFN- γ Kit." *Meso Scale Discovery*. © 2017 MESO SCALE DIAGNOSTICS, LLC, n.d. Web. 03 Aug. 2017.