STUDIES TO ELUCIDATE THE EFFECTS OF IRON(III) ON RECOGNITION OF HYALURONATE BY THE CELL SURFACE RECEPTOR CD44

By

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Director: Dr. Jack S. Summers, Professor of Chemistry
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Abstract

STUDIES TO ELUCIDATE THE EFFECTS OF IRON(III) ON RECOGNITION OF HYALURONATE BY THE CELL SURFACE RECEPTOR CD44.

Brittania J. Bintz, M.S.

Western Carolina University (August 2006)

Director: Dr. Jack S. Summers

Iron and the poly-anionic carbohydrate, hyaluronate (HA), have both been implicated as possible contributors to rheumatoid arthritis (RA), an autoimmune disease of unknown etiology. In this work, we hypothesized that in RA patients, iron is bound by HA in such a way as to affect HA binding by the cell surface glycoprotein CD44, a known HA receptor. To test this hypothesis, we developed cell based assays to determine the effects of iron binding to HA on HA binding by CD44. As part of this effort, we prepared biotinylated HA and biotinylated Fe complexed HA. Mesothelioma cells expressing CD44 were treated with each of these conjugates, and the resulting materials were treated with avidin-fluorescein and the bound fluorescein was measured using a fluorescence microtiter plate reader. We found that Fe (III) decreases the apparent affinity of CD44 receptors on Mesothelioma cells for HA (Observed fluorescence intensity decreased by a factor of ~2). In competition experiments, addition of non-biotinylated Fe complexed HA did not inhibit binding of biotinylated HA. Addition of non-biotinylated HA, however, appeared to increase the uptake of biotinylated Fe

complexed HA. These results suggest that iron(III) may crosslink HA molecules, leading to a greater uptake of HA. This crosslinking may influence receptor clustering which is believed to be important to intra-cellular signaling.

1. Introduction

1.A. Rheumatoid Arthritis

In general, inflammation results in an accumulation of fluid at a particular site in response to stimuli such as infection or trauma[1]. This fluid contains leukocytes, in particular lymphocytes[2]. As a result of inflammation, localized pain, redness, warmth, and swelling can occur[2]. Chronic inflammation causes degradation and digestion of the extracellular matrix[2].

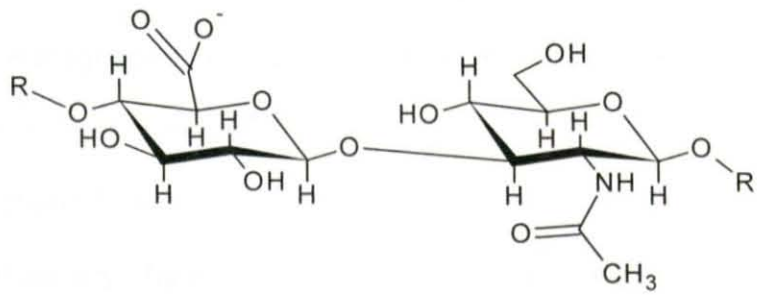
Rheumatoid arthritis is a chronic inflammatory disease principally affecting the articular cartilage of synovial joints[3]. The etiology of RA is unknown, but progression of the disease involves autoimmunity[3]. Symptoms of the disease include mild to severe pain and inflammation of synovial joints, decreased energy levels, and difficulty moving affected joints[3,4]. In RA, inflammatory fluids are high in T-cell concentration and accumulate within synovial joints in response to some unidentified trigger[1,5]. T-cells stimulate the production of cytokines, which then stimulate synovial cells to release matrix metalloproteinases that cause degradation of the joint cartilage[30].

1.B. Glycosaminoglycans

Proteoglycans are complexes of small proteins covalently linked to polysaccharide units. These saccharide polymers are commonly called glycosaminoglycans (GAGs)[5]. GAGs are composed of a repetition of specific saccharide pairs; essentially a coupling of a hexosamine such as n-acetylglucosamine or n-galactosamine to a hexauronic acid[7,6]. The structure of one such GAG, hyaluronic acid, is presented in Figure 1. Glycosaminoglycans are found in the extracellular matrix of articular cartilage and as cell surface polysaccharides[7]. The GAG hyaluronic acid (HA) is the predominant GAG found in cartilage and is also a major constituent of synovial fluid, a viscous, lubricating liquid present in the joints that contains a myriad of carbohydrates, proteins and lipids[8]. The viscosity and shock absorbing character of GAG solutions is due in part to the highly negative charge and high molecular weight of GAGs. These characteristics cause an attraction of water molecules to the surface of the polymer[9]. Conversely, water molecules are excluded from within the GAG matrix[7,10].

Unlike many GAGs, HA contains no sulfate[7]. HA is a linear carbohydrate consisting of repeating disaccharide units[7]. The repeating saccharides, namely glucuronic acid and n-acetyl glucosamine, are linked by beta 1,3 and beta 1,4 glycosidic likages as shown in Figure 1[6,7,16].

Figure 1. Structure of the Repeating Disaccharide Unit of HA.
HA is a carbohydrate composed of n-glucuronate (left saccharide) and n-acetylglucosamine (right saccharide) linked by a beta 1,3 glycosidic linkage.



1.C. Depolymerized HA in Synovial Joints

Research has indicated that the hyaluronic acid found in the synovial joints of rheumatoid arthritis patients is present in low concentrations and is of low molecular weight[9]. It has been proposed that the fragmented HA contributes to the self antigenic nature of rheumatoid arthritis[18]. Noble et al. have tested the ability of degraded and full length HA to trigger the response of NF-κB, a transcription factor that becomes activated during inflammation. They found that only low molecular weight HA elicits a response, although the mechanism is not known[18]. Additionally, McKee et al. discovered a myriad of inflammatory genes in macrophages that become activated when low molecular weight HA fragments accumulate[10]. These authors suggest that to maintain the cycle of inflammation in RA, a constant supply of fragmented HA must be generated[10].

1.D. Anemia in Rheumatoid Arthritis

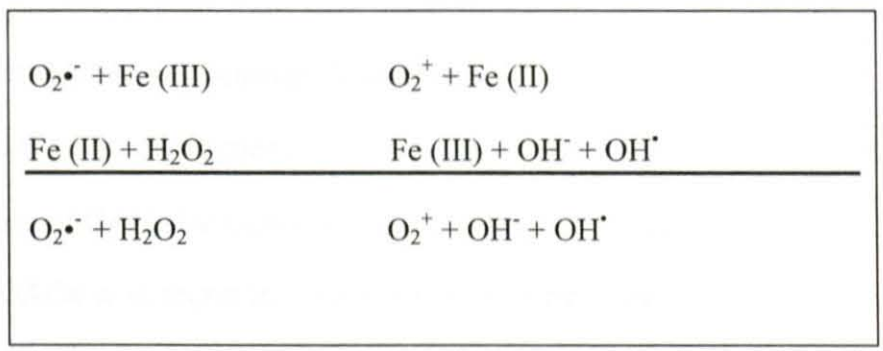
Systemic anemia generally accompanies RA. Anemia of chronic disease (ACD) is the most common cause for anemia in RA[22]. Approximately 30-70% of RA patients are diagnosed with ACD[22]. Although iron is present in low concentrations in the serum of RA patients due to ACD, the synovium contains high concentrations[24]. Iron in the synovium is bound to ferritin, an iron storage protein[24]. Additionally, there is a difference in the concentrations of iron bound to ferritin in the synovial fluid and the synovial membrane[24]. The lower concentration of saturated ferritin in the synovial fluid is contributed to exposure of the fluid to damaging superoxide free radicals and subsequent release of the iron from the ferritin[24].

1.E. Haber-Weiss Generation of Free Radicals

One theory that accounts for the degradation of HA in the joints is based on the observation that HA is degraded by reaction with hydroxyl radicals. These radicals are proposed to be generated by Haber-Weiss reaction of reduced iron with hydrogen peroxide. Because iron is known to be present in high concentrations in the synovium of rheumatoid arthritis patients, it has long been suspected that damaging free radicals are generated by the Haber-Weiss reaction[11,12,24]. The Fe (III) present in the joints reacts with superoxide ($O_2 \cdot^-$) giving rise to dioxygen and a reduced Fe (II) molecule that can react with hydrogen peroxide. When the Fe (II) reacts with hydrogen peroxide, a highly

reactive hydroxyl radical forms. The hydroxyl radical reacts rapidly with many biomolecules it encounters, leading to carbohydrate damage, membrane lipid peroxidation, protein crosslinking, proteasomal degradation, and DNA fragmentation[15]. This may lead to depolymerization of the matrix of joint cartilage.

Figure 2. Haber-Weiss Reaction.



1.F. CD44 Cell Surface Glycoproteins

CD44 is a cell surface glycoprotein that is involved in many cellular functions including cell communication, signal transduction, extracellular matrix assembly, and cell adhesion and migration[1, 25]. CD44 is principally expressed on B cell and T cell lineages. Furthermore, CD44-hyaluronan interactions may function in cell-to-cell communication and in cell adhesion because of the high viscosity of the hyaluronan ligand[25, 26]. Due to its high expression in tumor cells and its role in cell migration, CD44 has been implicated in metastasis[25]. For this project, mesothelioma cells were chosen for their high degree of CD44 expression[27].

The synovial fluid in RA patients contains both CD44 cell surface glycoproteins and hyaluronan[9]. When CD44 is associated with the HA ligand, normal cellular function is observed[28]. When the association between HA and CD44 is disrupted, complications arise and apoptotic pathways are induced[28]. This disruption is proposed to play a central role in the autoimmune nature of rheumatoid arthritis.

1.G. Role of GAGs in Rheumatoid Arthritis

Recent research suggests that rheumatoid arthritis is mediated specifically by recognition of GAGs by CD44 and CD4 T-cells[5,2]. In addition, an autoimmune reaction to GAGs was found to cause RA symptoms in synovial joints of mice, which correlated with the prevalence of CD4 T-cells expressing GAG-binding cell-surface proteins[5]. It has been established that CD44+ cells mediate binding with GAGs and are found in the synovial fluid of RA patients but not healthy patients[1,6]. A study conducted by Wang et al. included the use of an aluminum hydroxide ($Al(OH_3)$) adjuvant that was found to exacerbate RA symptoms in the mouse models[5]. The role of this adjuvant was not considered in their results.

1.H. Hypotheses

Because of their net negative charge, GAGs are able to bind to metal cations, presumably causing a conformational change of the complex. We propose that this change affects the recognition of GAGs by CD44 and may potentially have serious consequences related to rheumatoid arthritis. The role of the metal ion in CD44 recognition has not been studied previously. Described in this thesis is the development of methods to test whether the metal ion contributes to this decrease in recognition.

Also described in this thesis is the development of a method to depolymerize HA. Depolymerized hyaluronic acid is found within the joints of rheumatoid arthritis patients. Depolymerized HA fragments may cause a rapid autoimmune response, creating a symptomatic cascade of events. Additionally, iron present in the joints of rheumatoid arthritis patients may preferentially bind lower molecular weight HA. This favored binding may increase the inflammatory response and antigenic recognition in rheumatoid arthritis.

2. Experimental

2.A. Limit of Detection Determination for Microtiter Plate Reader

A limit of detection of the fluorescence microtiter plate reader (FLUOstar Optima) was established. A 5 µg/mL solution of avidin-fluorescein was prepared and serially diluted 3:1 to a concentration of 0.05 µg/mL. HEPES buffer (1.5 M NaCl, pH 8.5, 0.08% sodium azide) was used as the diluent. Samples were prepared, transferred to a 96-well microtiter plate, and analyzed in triplicate using an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The data from three experiments were averaged, and the fluorescence intensities were plotted against avidin-fluorescein concentration to give a linear relationship. The signal to noise ratio was considered in determining the lowest detectable limit. The limit of detection was experimentally determined to be 0.5 µg/mL of avidin-fluorescein.

2.B. Cell Culturing

2.B.1. Preparation of Cell Culture Lab

Animal cell culture requires specific conditions for the successful propagation of contaminant-free cell populations. A properly working microbiological safety cabinet, certified CO_2 incubators, and decontaminated workspace are only some of things necessary for cell culture. When this project

was in its initial stages, a cell culture laboratory did not exist at Western Carolina University. Initially, a lab was sterilized using an antibacterial alkaline detergent and an antifungal solution. All exposed laboratory surfaces were decontaminated with both cleaning solutions. The microbiological safety cabinet was inspected and a new HEPA filter and UV light were installed. New CO_2 incubators were installed and calibrated.

2.B.2. Initial Cell Subculturing

Human mesothelioma cells from ATCC (ATCC# CRL-5946; NCI# H2452) were maintained in the vapor phase of liquid nitrogen to prevent loss of viability until the culture was initiated as indicated by the supplier. Media used for culturing was an RPMI 1640, which contains L-amino acids, vitamins, minerals and buffers [13]. The media was supplemented with 2mM L-glutamine as a carbon source adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES buffer, and 1.0 mM sodium pyruvate (90%).

The protocol recommended by the supplier for initial subculture was used. The hood was sterilized by swabbing all surfaces with 70% ethanol. All supplies were moved into the hood following sterilization with 70% ethanol. Media was supplemented with 10% FBS and 1% penicillin-streptomycin for the prevention of bacterial contamination. The supplemented media (15 mL) was then transferred to a T75 vented flask and incubated at 37°C for 15 minutes to assure that the media was at the proper temperature and pH. The cells were then thawed

rapidly to 37°C and the contents of the vial were added to the T75 flask making a final total volume of 16 mL. The flask was incubated at 37°C with 5% CO_2. The cells were viewed daily using an inverted light microscope to monitor cell growth and confluence. The media was observed to check for signs of contamination, including turbidity, slow cell growth, and the presence of microbial colonies. The media contained phenol red pH indicator with a pH range of 6.8 – 8.0 and appears yellow at acidic pH and fuchsia when basic. When a pH change was observed, the old media was removed and new media was added.

2.B.3. Cell Passage

Cell passage or subculturing is defined as the division of a cell culture[29]. Cells are passaged at 80% confluence, or when the available growth substrate is almost completely occupied and nutrients are becoming scarce[29]. The cell passage protocol for human mesothelioma cells was as follows. Initially, old culture medium was removed[29]. Cells were scraped from the culture substrate using a cell scraper. Scraping was chosen rather than enzymatic methods to preserve the CD44 cell surface receptor. Six to eight mL of media was added to the flask and the solution was gently pipetted to disperse the cells. Aliquots of the suspension were then transferred into sterile T75 flasks at subcultivation ratios of 1:4 or 1:6 and incubated at 37°C. A complete change of media was conducted every 1 to 2 days.

2.B.4. Cryopreservation of Cells

To safeguard against the fact that something might happen to the incubating cell population, stocks of additional healthy cell populations were stored cryogenically. Cells were fed one day prior to freezing to prevent unnecessary additional stress. Prior to cryopreservation, the cells were viewed using an inverted light microscope and inspected for signs of contamination. The media was removed and the cell layer was washed with 5 mL of Hanks Buffered Saline Solution (HBSS) to wash the cell layer for removal of traces of serum that could inhibit trypsin. Subsequently, 5 mL of trypsin-EDTA (at 37°C) was added to the flask. The culture was observed under an inverted light microscope for cell rounding and phase change, indications that the cells were no longer attached to the flask surface. This process typically took no longer than 5 minutes and was not allowed to proceed beyond 15 minutes because cell damage could occur. After 5 minutes, 5 mL of media was added and the cells were collected in a 15 mL conical centrifuge tube. Next, a 200 µl aliquot of the cell suspension was removed for cell counting (see protocol below). The 15 mL conical tube was then centrifuged in a Sorvalll RC 5C at 100 x g at 4°C for 5 minutes. The supernatant was discarded and the pellet was resuspended in enough cryoprotective media to give a final concentration of 1-2 x 10^6 cells/mL. The cryoprotective media was composed of the original media supplemented with 10% DMSO. The cell suspensions were then transferred into cryovials and cooled at a rate of 1°C per minute until a final temperature of -80°C was reached.

2.B.5. Cell Counting

A solution was prepared that contained 0.2 mL of cell suspension (as described above), 0.5 mL of trypan blue (0.4%) and 0.3 mL of HBSS. The solution was incubated at room temperature for 5 to 15 minutes. During this incubation trypan blue stained the dead cells and was excluded from the live cells. The solution was then transferred to a hemacytometer and viable cells were counted using a protocol supplied by Sigma-Aldrich.

2.C. Preparation of Samples for Competition Assays

The following samples were prepared in order to study the effects of iron (III) on recognition of HA by its cell surface receptor CD44: (1) HA, (2) Fe complexed HA, (3) biotinylated HA, and (4) biotinylated Fe complexed HA.

2.C.1. Preparation of HA

A solution of hyaluronic acid was prepared by adding 0.2 g Na-HA to a 70:30 mixture of 95% ethanol/HCl (0.1M) and stirring for 1 hour. The solution was then centrifuged for 5 minutes. The supernatant was removed and discarded. This step was done to convert the Na-HA to hyaluronic acid. The converted hyaluronic acid (1.5 mg) was then added to deionized water (1.0 mL) and stirred overnight to assure complete dissolution.

2.C.2.a. Preparation of Fe Complexed HA: Conversion of Sodium Hyaluronate to HA. Preparation of the HA-Fe complex (HA:Fe ratios of 1:1, 2:1, 4:1, 8:1) included a step in which sodium hyaluronate was converted to hyaluronic acid. Sodium hyaluronate (0.6 g) was added to 50 mL of a 70:30 mixture of 95% ethanol / HCl solution (1.0 M, pH 2.0) and allowed to stir at room temperature for 15 minutes. The solid was filtered off and washed with the remaining mixture of 70:30 ethanol / 0.1 M HCl and 95% ethanol (25 mL). The samples were then dried in vacuo. A stock solution was prepared using the dried hyaluronic acid (0.51 g) dissolved in 17 mL of deionized water. The sample was heated in a water bath (60°C) to assure complete dissolution. Four samples were then prepared with HA repeating unit to Fe molar ratios of 1:1, 2:1, 4:1, and 8:1. $Fe(NO_3)_3 \cdot 9H_2O$ (0.1 M, 76.5 µL) was added to each reaction tube containing 1, 2, 4 and 8 µL of HA solution respectively. $Fe(NO_3)_3 \cdot 9H_2O$ was chosen for its ease of solubility. According to Mercê et al. a scant precipitate should have been evident upon addition of the $Fe(NO_3)_3 \cdot 9H_2O$[Error! Bookmark not defined.]. Unlike what was described by Mercê et al., no orange gelatinous precipitate was recovered. This preparation was considered unsuccessful.

2.C.2.b. Preparation of Fe Complexed HA: Stirring Schedule. An additional trial to prepare an Fe complexed HA sample included a stirring schedule for the conversion of sodium hyaluronate to hyaluronic acid to determine if the duration of conversion affected the outcome of the synthesis of the HA-Fe. Hyaluronic

acid (0.04 g) was added to 10 mL of a 70:30 mixture of 95% ethanol /HCl (0.1 M). The solutions were stirred for periods of 5 minutes, 1 hour, 5 hours, 8 hours, and overnight. The solutions were then centrifuged for 15 minutes and the supernatant was removed. The solid was then washed with 95% ethanol/HCl (0.1 M) and dried under a vacuum. The remaining solid was transparent and adhered to the side of the conical centrifuge tube. Deionized water was added and the tube was placed in an 80°C water bath for dissolution of the solid. The solid did not dissolve in water. Since hyaluronic acid is water soluble and Fe_2O_3 is not, this trial was aborted because insolubility in water suggested that the product did not contain HA.

2.C.2.c. Preparation of Fe Complexed HA: Modified Protocol. A slightly different protocol from Mercê et al. was used in this trial, which proved to be successful[14]. Hyaluronic acid (0.1 g) was added to acidified deionized water (33 mL, pH 4.0 adjusted with HNO_3) and stirred for 5 minutes. This step allows for the conversion of the Na-HA to hyaluronic acid. Aqueous $Fe(NO_3)_3 \cdot 9H_2O$ (283 µL, 0.1M) was added to the solution. Immediately a large amount of opaque, gelatinous, threadlike precipitate formed. The pH was adjusted once again to 3.0 using HNO_3 to prevent precipitation of the Fe and to facilitate dissolution of the remaining solid Na-HA. As the pH was reduced the gelatinous precipitate dissolved. The solution was stirred for approximately 1 week. The solution was then buffered to a physiological pH (6.5 – 7.2) using PIPES buffer (0.6 g PIPES,

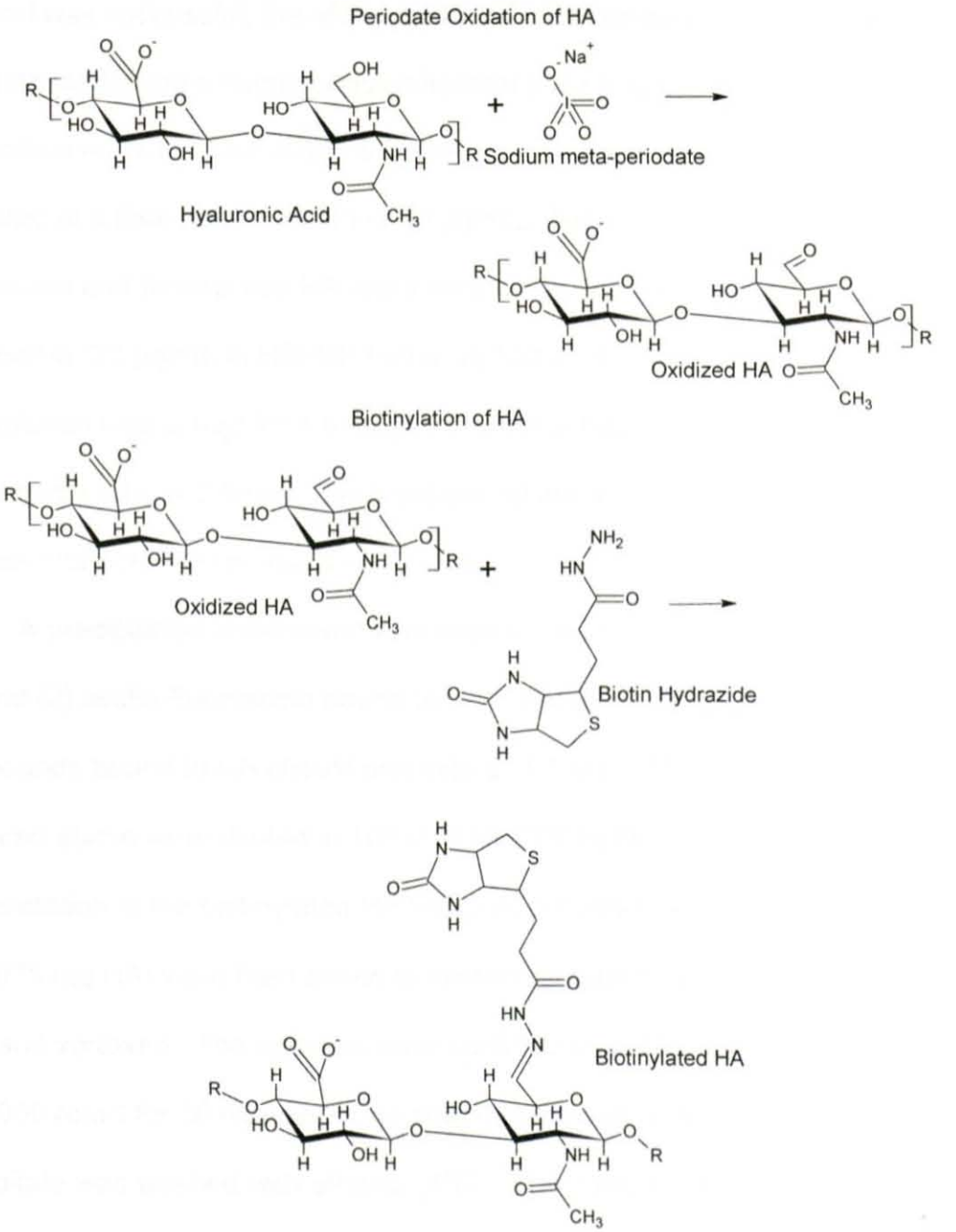
3 mL NaOH). Upon addition of the PIPES the solution turned clear yellow with no visible precipitate. Ethanol was then added to precipitate the complex. A total of 250 mL of ethanol was needed for precipitation. The precipitate was gelatinous and yellow. The sample was stored at -20°C overnight to allow complete precipitation of the complex. The solution was then divided into 15 mL conical centrifuge tubes and centrifuged for 30 minutes (with a Sorvall HS-3000 rotor). The supernatant was removed and discarded. A total of 0.1193 g of product was recovered. A small amount of the solid (2.5 mg) was added to 1.5 mL of deionized water to determine if the solid was soluble in water. If the solid was not soluble it may suggest that it was Fe_2O_3. The solid was soluble in water and the solution appeared light yellow in color.

For characterization, 0.062 g of solid was dissolved in 1.5 mL deionized water. The solution was placed in a 0.45 µm microspin column and centrifuged for 10 minutes at 10,000 rpm. The resulting filtrate appeared to be yellow suggesting that the Fe passed through the filter, which indicated that it was bound by the carbohydrate. If the Fe was present as Fe_2O_3 it would not have passed through the filter. To further characterize the complex, the filtrate was placed in a 10,000 MWCO microspin column and centrifuged for 10 minutes at 10,000 rpm. The retentate was yellow in color and the filtrate was clear. This suggested that the Fe did not pass through the column and was therefore attached to the high molecular weight carbohydrate which was unable to pass though the filter. UV-vis analysis results showed maximum absorbance for

$Fe(NO_3)_3$ at approximately 285 nm, while the Fe complexed HA did not display a similar maximum absorbance. Additionally, Fe complexed HA (1.5 mg) was dissolved in deionized water (10 mL) and analyzed for iron content using ICP-OES. ICP results showed that a 1.5 mg sample contains 0.01762 mg of iron.

2.C.3.a. Preparation of Biotinylated HA. HA was biotinylated using commercially available EZ-link biotin hydrazide (Pierce) according to the supplier's protocol (Figure 3). First, a carbohydrate solution was prepared by dissolving hyaluronic acid in ammonium acetate coupling buffer (2.0 mg/mL; pH 5.5). Cold sodium metaperiodate (20 mM; 1 mL) was added to the solution of hyaluronic acid (1 mL) and the oxidation reaction was allowed to proceed for 30 minutes at 4°C in the dark. Glycerol was added to the reaction mixture at a final concentration of 15 mM to stop the oxidation. The resulting solution was dialyzed overnight against ammonium acetate coupling buffer using a Slide-A-Lyzer dialysis cassette 10K MWCO (Pierce). Then, biotin hydrazide was added at a final concentration of 5 mM and stirred at room temperature for 2 hours. The hydrazide group reacts with any carbonyl groups formed during the oxidation reaction to form the linkage. Following the incubation, the biotinylated molecule was separated from unreacted biotin hydrazide using an additional overnight dialysis.

Figure 3. Periodate Oxidation and Biotinylation of Hyaluronic Acid (HA).



2.C.3.b. Characterization of Biotinylated HA. To ensure that the synthetic protocol was successful, the aforementioned biotinylated HA samples were characterized using a fluorescence microtiter plate reader. A solution of avidin-fluorescein in HEPES buffer (0.15 M NaCl, pH 8.5, 0.08% sodium azide) was prepared at a final concentration of 20 µg/mL. Then, a solution of avidin-fluorescein and biotinylated HA was prepared by adding 100 µl of avidin-fluorescein (20 µg/mL in HEPES buffer) to 100 µl of biotinylated HA (1.5 mg/mL). The solution was stirred for 1 hour at room temperature. This protocol was performed a total of 3 times. Each solution of avidin-fluorescein with biotinylated HA was characterized as described below.

A precipitation experiment was used to confirm that (1) biotin was linked to HA and (2) avidin-fluorescein bound to biotinylated HA. Only HA and compounds bound to HA should precipitate. Intially, 100 µl of the samples prepared above were diluted in 100 µl of HEPES buffer to equal the concentration of the biotinylated HA with avidin-fluorescein. The samples (100 µl; 0.075 mg HA) were then added to microcentrifuge tubes with ethanol (1 mL, 4°C) and vortexed. The samples were centrifuged at 10,000 rpm (with a Sorval SH-3000 rotor) for 30 minutes. The supernatant was removed and the remaining precipitate was washed with ethanol (4°C). The samples were centrifuged once again and the supernatant removed. The resulting precipitates were reconstituted in HEPES buffer (500 µl; 0.15 M NaCl, pH 8.5, 0.08% sodium azide). Avidin-fluorescein that did not undergo the precipitation protocol was

used as a control, as well as a sample of avidin-fluorescein that did undergo the precipitation reaction. The samples and controls were analyzed using a microtiter plate reader.

Samples of precipitated biotinylated HA with avidin-fluorescein and the untreated avidin-fluorescein control were expected to give high fluorescence intensities. The results of this procedure were consistent with the presumption that biotin was indeed conjugated to the HA. Fluorescence was observed in the wells containing biotinylated HA with avidin-fluorescein and also in the wells containing the untreated avidin-fluorescein. The wells containing avidin-fluorecein did not show fluorescence, indicating that this sample was not precipitated by ethanol. These results indicate that the compound synthesized was actually biotinylated HA. Additionally, this experiment also proved that biotin, when conjugated to HA, was able to bind to avidin-fluorescein in solution. The ratio of biotin to HA repeating unit was not determined.

2.C.4. Preparation of Biotinylated Fe Complexed HA

A complex of biotinylated Fe complexed HA was synthesized. Biotinylated HA (prepared as described above) (3 mL, pH 5.5) was added to solid $Fe(NO_3)_3 \cdot 9H_2O$ (1.5 mg/mL). After vortexing, the pH was adjusted to 7.0 using PIPES buffer (117 µL) and the solution was stirred overnight at 37°C. A heavy orange precipitate formed. The solution was centrifuged through a 0.45 µm filter. Since Fe_2O_3 cannot pass through a 0.45 µm filter, it was assumed that the light

yellow filtrate contained the Fe complexed biotinylated HA and not Fe_2O_3. The sample was analyzed for iron content using ICP-OES. A sample was prepared for ICP-OES by diluting 100 µl of the Fe complexed biotinylated HA in 10 mL of deionized water. ICP results suggested that the iron content of the sample was below the limit of detection of the instrument. However, because the solution was yellow in color, iron was present.

2.D. Assay Development: Detection of CD44 Binding of HA

2.D.1. Fluorescence Quenching by Fe (III) Assay

Potentially, the fluorescence of fluorescein could be quenched by Fe bound to HA. Samples were prepared that contained a consistent concentration of avidin-fluorescein (5 µg/mL, 6 µl) but varying concentrations of biotinylated HA and biotinylated Fe complexed hyaluronic acid. Reagent volumes are listed in Table 1. The total reaction volume was 150 µL per well. Reagents were placed into wells of a black microtiter plate for reduction of fluorescence crosstalk between wells. Analysis of fluorescence signal was accomplished using a fluorescence microtiter plate reader. All samples were read in duplicate. If quenching occurred, fluorescence would decrease as the concentration of biotinylated Fe complexed HA increased.

Table 1. Reagents Added for Fluorescence Quenching by Fe (III) Assay.

Sample	Avidin Fluorescein (µL)	HA*-Fe [1.5 mg/mL] (µL)	HA* [1.5 mg/mL] (µL)	HEPES Buffer (µL)
Blank	0	0	0	150
Positive Control	6	0	0	144
Sample #1	6	40	0	104
Sample #2	6	20	20	104
Sample #3	6	10	30	104
Sample #4	6	5	35	104

*Denotes biotinylation of the sample.

2.D.2. Confirmation of CD44 Expression Using 1° and 2° Antibodies

Antibodies specific to CD44 cell surface glycoproteins were used to confirm the expression of the extracellular CD44 molecule. Mesothelioma cells were seeded into a white 24 well microtiter plate at 5000 cells per well. The cells were allowed to incubate for 24 hours at 37°C with 5% CO_2. The 24 hour incubation allowed for re-expression of the CD44 molecule in the event that damage to the receptor had occurred during cell passage, while maintaining a relatively low confluence. High cell confluence in fluorescence assays gives a false low fluorescence signal. A primary mouse monoclonal antibody (abCam, ab6337) to CD44 was diluted 1:100 in PBS with 3% BSA and incubated with the mesothelioma cells for 20 minutes at room temperature. The cells were then washed three times, for 3 minutes per wash, in PBS with 3% BSA. A secondary rabbit polyclonal antibody to mouse IgG conjugated to Texas Red was diluted1:100 in PBS with 3% BSA. The cells were incubated with the secondary antibody for 15 minutes at room temperature, and then washed three times in PBS. The fluorescence was detected at an excitation wavelength of 584 nm and

an emission wavelength of 612 nm using a fluorescence microtiter plate reader. A slight increase in fluorescence was detected and an additional incubation with a 1:10 dilution of secondary antibody was conducted for 15 minutes at room temperature. The fluorescence signal increased slightly, indicating that the primary antibody was able to bind to the CD44 molecule. Based on the slight increase in fluorescence, it was believed that the incubation times weren't sufficient enough to allow complete association of the CD44 molecules with the antibodies. In future assays the incubation times were increased to 30 minutes as indicated by Harlow et al.[20] The cells were then incubated for 30 minutes at room temperature with Hoescht dye. Hoescht dye binds DNA at the minor groove and has a fluorescence maximum at 460 nm when bound to DNA. Hoescht staining provides evidence for continuity in cell confluence among the wells.

2.D.3. Confirmation of CD44 Expression by Antibody Blocking

Primary antibodies were employed to verify that CD44 was being expressed on the surface of the mesothelioma cells and that hyaluronic acid was binding to the CD44 receptor. An anti-CD44 mouse monoclonal antibody (abCam, ab6337) was chosen for its specificity to the CD44 receptor. No literature exists on the epitope of this antibody. If the antibody does bind to the receptor directly it may block any subsequent HA from binding. If this is the case, upon addition of biotinylated HA to cells that had been pre-incubated with

CD44 antibody, a reduction in the fluorescence signal would be observed compared to cells incubated with only biotinylated HA. These results would indicate that CD44 is being expressed by the cells and that both the antibody and the biotinylated HA are binding to the same region of the receptor.

Mesothelioma cells were seeded into a white, 96 well microtiter plate at 500 cells per well. The cells were allowed to incubate at 37°C with 5% CO_2 for 24 hours. The anti-CD44 antibody was diluted 1:100 in PBS with 3% BSA. The diluted antibody (40 µl) was added to the cells and incubated for 30 minutes at room temperature. The cells were washed 3 times for 3 minutes in PBS with 3% BSA (250 µl). Biotinylated HA (1.5 mg/mL, 40 µl) was added to the wells and incubated for 30 minutes at 37°C, and washed in PBS with 3% BSA (250 µl, x3 for 3 minutes each wash). Additionally, wells were incubated with biotinylated HA and no antibodies for use as a comparison of fluorescence signal. The cells were then fixed by incubating in paraformaldehyde (40 µl; 4 %) for 10 minutes at room temperature. The wells were washed with PBS with 3% BSA (250 µl, x3 for 3 minutes each wash). Fixing did not alter the morphology of the CD44 receptor as seen in section 2.D.4.b. All of the wells were incubated for 30 minutes at 37°C with avidin-fluorescein (20 µg/mL, 40 µl), and washed in PBS with 3% BSA. The fluorescence intensity was detected using a fluorescence microtiter plate reader. The cells were then stained with Hoescht dye for confluence analysis.

2.D.4.a. Depletion Assay with Live Cells. An assay was conducted in which a depletion in the fluorescence signal of avidin-fluorescein after incubation with mesothelioma cells was compared to the increased fluorescence signal of the cells. This data helped determine the extent of avidin-fluorescein binding to the biotinylated HA and whether the fluorescence signal was high enough above background to be detected. Mesothelioma cells were seeded into a white, 96 well microtiter plate at 500 cells per well. The cells were allowed to incubate for 24 hours at 37°C with 5% CO_2. The media was removed and the cells were washed with PBS. Biotinylated HA (1.5 mg/mL, 40 µl) was added to the wells and allowed to incubate for 30 minutes at 37°C. The cells were washed with PBS. Avidin-fluorescein (20 µg/mL, 40 µl) was added and the cells were allowed to incubate for an additional 30 minutes at 37°C. The avidin-fluorescein from the well which was initially incubated with biotinylated HA was then transferred to an empty well and fluorescence was analyzed using a fluorescence microtiter plate reader. The cells were then incubated with Hoescht dye for 30 minutes at room temperature for confluence analysis.

If avidin-fluorescein is bound to biotinylated HA and the HA is bound to CD44, the fluorescence of the transferred avidin-fluorescein should be lower than the fluorescence of a stock solution of avidin-fluorescein. As a control, avidin-fluorescein was added and then removed from a well which was then washed to demonstrate that the fluorescence signal was not due to traces of avidin-fluorescein left in the well after washing. It was evident from the data that the

washing protocol was sufficient. Additionally, an increase in fluorescence in the well that was incubated with biotinylated HA and avidin-fluorescein was expected. The results indicated that avidin-fluorescein was binding to biotinylated HA, which was bound to CD44 expressed by the mesothelioma cells.

2.D.4.b. Depletion Assay with Fixed Cells. Because subsequent assays were lengthy, cell viability was a concern. One method used to circumvent questionable viability during a cell-based assay is cell fixation. Two different techniques for cell fixing include the use of organic solvents and the use of formaldehyde and its derivatives for protein crosslinking[20]. In general, methods that employ organic solvents tend to dehydrate the cell, altering lipid composition[20]. Crosslinking agents like paraformaldehyde maintain the cellular morphology but may modify epitope regions of receptors[20]. However, modification can be reversed by incubation with proteases[20]. For this assay, paraformaldehyde was chosen because of the low potential for morphological modification. During cell fixation with paraformaldehyde, extracellular protein matrices become cross-linked and "fixed" in place while carbohydrates, lipids and nucleic acids remain virtually unaltered[20].

Preparation of paraformaldehyde included dissolution of the solid (0.8 g) in deionized water (10 mL) with NaOH at 60°C. Dissolution of paraformaldehyde results in an aqueous solution of methylene hydrate which reacts chemically in the same manner as gaseous formaldehyde in cell fixing[19]. The solution was

cooled to room temperature and 2X PBS was added (10 mL). This depletion assay was conducted similar to the initial depletion assay with the exception that the cells were fixed prior to incubation with avidin-fluorescein. The fixing step included incubation of the cells in paraformaldehyde (40 µl, 4% solution) for 10 minutes at room temperature. After the fluorescence was analyzed using a microtiter plate reader, the cells were then stained with Hoescht dye for analysis of cell confluence. Additionally, these assays provided insight into the optimization of the cell based assay. Incubation times, wash steps, and the fixing protocol were optimized.

2.D.5. Competition Assay

Competition experiments were conducted to determine the effects of Fe on HA binding by CD44. In these experiments, the competing reactants (ie, either Fe complexed HA and biotinylated HA, or HA and biotinylated Fe complexed HA) were added to microtiter plate wells containing the mesothelioma cells. The uptake of the biotinylated reactant was measured by its ability to bind the fluorescently labeled avidin. Experiments in this series are described below.

A competition assay was conducted to determine if metal complexed HA binds to CD44 receptors with less affinity than HA. Because HA molecules are polyanions, the presence of the metal cation could cause crosslinking among normally linear HA chains. Two crosslinking reactions were conducted in which biotinylated HA and Fe complexed HA were added to a microcentrifuge tube and

incubated for 30 minutes at room temperature. Similarly, HA and biotinylated Fe complexed HA were added and incubated in the same manner.

2.D.5.a. Characterization of HA Binding. The binding patterns of biotinylated HA and biotinylated Fe complexed HA were each measured in separate experiments. In these experiments 40 μL aliquots of the two reactants (1.5 mg/mL) were separately added to wells containing mesothelioma cells and the solutions were incubated for 30 minutes at 37°C.

2.D.5.b. Competition Reactions. Competitive reagent samples were prepared in two ways: either by simultaneously mixing the reagents with the mesothelioma cells, or by mixing the competitive reactants and allowing them to incubate for 30 minutes prior to their introduction to the cells. In a typical competition experiment, biotinylated HA (1.5 mg/mL, 40 μl) and Fe complexed HA (1.5 mg/mL, 40 μl) were added to a well simultaneously, as were HA (1.5 mg/mL, 40 μl) and biotinylated iron complexed HA (1.5 mg/mL, 40 μl).

2.D.5.c. General Procedures. All reactions were conducted in duplicate. Mesothelioma cells were seeded into a white, 96 well microtiter plate at 500 cells per well and incubated at 37°C at 5% CO_2 for 24 hours. The crosslinked samples were added to separate wells of the microtiter plate. Following incubation with a biotinylated reactant, the reagents were removed and the cells

were washed in PBS (x3 for 3 minutes each wash). The cells were then incubated in 4% paraformaldehyde (40 µl) for 10 minutes at room temperature for fixing. The paraformaldehyde was removed and the cells were washed again. Avidin-fluorescein (20 µg/mL, 40 µl) was added to each well and allowed to incubate for 30 minutes at room temperature. Again, the reagent was removed and the cells washed. PBS (40 µl) was added to each well. The fluorescence was detected using a microtiter plate reader. The cells were then stained with Hoescht dye for confluence analysis.

2.E. Depolymerization Studies

2.E.1. Current Work with Depolymerized HA

The depolymerization of HA and the effects in rheumatoid arthritis is currently under investigation in the laboratories of Jack Summers and Lori Seischab. For this thesis project, a protocol was developed for depolymerization of HA using chemical and enzymatic methods. The project was assumed by Carmen Batchelor, who is currently seeking to quantify saccharide chain length of the degraded HA using electrospray ionization mass spectrometry (EI-MS).

2.E.2. Depolymerization of HA

Two different methods were used to depolymerize HA. Initially, several trials of a chemical digestion using HCl and heat was used. Digested samples were analyzed for extent of depolymerization using NMR and EI-MS. We were unable to provide sufficient chemical digestion by this method and have abandoned it in favor of an enzymatic method employing hyaluronidase. The depolymerized polysaccharides were analyzed by Jack Summers with nuclear magnetic resonance (NMR) and EI-MS.

2.E.3. Chemical Depolymerization of HA: Short Incubation and NMR Analysis

Solutions of HA were prepared by Lauren Rodgers. The solutions were placed in a 70°C water bath for durations of 6, 12, 24, 48, 72 and 96 hours. Different times were used to determine the length of incubation that gives the optimal range of depolymerization.

Reaction tubes of depolymerized HA (250 µL from 6 and 96 hour time aliquots respectively) were prepared for NMR F- relaxation studies. The original pH was 10 due to Na_2CO_3/$NaHCO_3$ buffer present in the solution. The solution was acidified to pH 2.5 by addition of HCl (62.5 µL, 1M) to prevent immediate precipitation of $Fe(NO_3)_3 \cdot 9H_2O$. $Fe(NO_3)_3 \cdot 9H_2O$ (0.1 M, 10 µl) was added to the solution at a final concentration of 1 mM. The solution was allowed to stir for 10 minutes at room temperature. Following the 10 minute reaction period, NaF (0.1M, 200 µl) was added at a final concentration of 20 mM. The solution was

brought to pH ~5 using succinate buffer and the total volume was brought up to 1 mL using deionized water. Samples were also prepared with Fe concentrations of 2.0 mM and 3.0 mM. The Fe was not soluble at higher concentrations. Table 2 shows the concentrations and quantities of the reagents for all NMR analyses. Additional samples were prepared for longer depolymerization.

Table 2. Reagent Concentrations and Quantities for F- Relaxation Studies.

REAGENT	1 mM Fe (conc./quantity)	2 mM Fe (conc./quantity)
$Fe(NO_3)_3 \cdot 9H_2O$	0.1M/10 µL	0.1M/22 µL
NaF	0.1 M/200 µL	0.1 M/220 µL
Depolymerized HA	250 µL	250 µL
HCl	1 M/ 62.5 µL	1 M/ 62.5 µL
Succinate Buffer	387.5 µL	445.5 µL
NaOH	90 µL	100 µL

2.E.4. Chemical Depolymerization of HA: Long incubation and NMR Analysis

An additional digestion was run to optimize the protocol for chemical depolymerization and for additional NMR relaxation analysis. Microcentrifuge tubes were prepared by dissolving 6 mg of HA in 1.5 mL of HCl (0.01 M). The tubes were incubated at 70°C for 0, 1, 2, 4, 8 and 16 days. Upon removal from the water bath the tubes were stored at -20°C. NMR reactions were prepared as described in the previous section. Samples were prepared with Fe concentrations of 0, 0.5, 1, 1.5 and 2.0 mM respectively. Table 3 shows the reagent concentrations and quantities for all reactions. The total reaction volume was 1 mL. The samples were then transferred to NMR tubes (560 µL) and D_2O

(60 µL) was added. After standing in NMR tubes overnight, each sample contained an orange precipitate. This observation suggested that the thermodynamic minimum was Fe_2O_3. NMR analysis was not conducted.

Table 3. Reagent Concentrations and Quantities for F- Relaxation Studies.

REAGENT	0 mM Fe (conc./ quantity)	0.5 mM Fe (conc./ quantity)	1 mM Fe (conc./ quantity)	1.5 mM Fe (conc./ quantity)	2.0 mM (conc./ quantity)
$Fe(NO_3)_3 \cdot 9H_2O$	0.1M/0 µL	0.1M/5 µL	0.1M/10 µL	0.1M/15 µL	0.1M/20 µL
NaF	0.1 M/200 µL	0.1 M/200 µL	0.1 M/200 µL	0.1 M/200 µL	0.1 M/200 µL
Depolymerized HA	250 µL	250 µL	250 µL	250 µL	250 µL
HCl	1 M/ 63 µL	1 M/ 63 µL	1 M/ 63 µL	1 M/ 63 µL	1 M/ 63 µL
Succinate Buffer	567 µL	562 µL	557 µL	552 µL	547 µL
NaOH	100 µL	100 µL	100 µL	100 µL	100 µL

No samples were analyzed due to insolubility of Fe.

2.E.5. Chemical Depolymerization of HA: Long Incubation and EI-MS Analysis

MIcrocentrifuge tubes were prepared that contained 6 mg of Na-HA and 1.5 mL of HCl (0.1 M). They were incubated in a 70°C water bath for varying time periods. The samples were digested for 5, 8, 10, 15 or 20 days. The 5 day sample was then lyophilized using a centrivap concentrator. The resulting solid was reconstituted in a buffer mixture of 10 µM NH_4OH: 10 µM formic acid. The solution was analyzed for extent of depolymerization using EI-MS. The spectra gave one large peak with a mass to charge ratio (m/z = 380) that was too low to be the dimer unit. We interpreted this result as indicating that the digestions were not sufficient to give products small enough for analysis by EI-MS.

2.E.6. Enzymatic Depolymerization using Hyaluronidase: Protocol Adapted from Saad et al.

Digestions were performed using bovine testicular hyaluronidase (Sigma-Algrich). The protocol for degradation was adapted from Saad et al. to determine if a protocol for bacterial hyaluronidase (from *Streptococcus dysgalactiae*) would be successful for bovine testicular hyaluronidase[21]. Microcentrifuge tubes were prepared at 1 mg/mL of HA as described by Saad et al[21]. A 15 µg aliquot of HA was added to 40 µL of sodium acetate buffer (pH 7.5) and 5 µU of hyaluronidase. The reaction was incubated at 37°C for 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours and 8 hours. The digestion was then quenched by adding 750 µL of methanol and 710 µL of water to the reaction tube. EI-MS analysis indicated that degradation of the HA did not occur. It was determined that the protocol was not sufficient for bovine testicular hyaluronidase. A higher concentration of the enzyme was necessary for depolymerization.

2.E.7. Enzymatic Depolymerization using Hyaluronidase: Protocol Adapted from Tawada et al.

A protocol for digestion of HA was adapted from Tawada et al[14]. Initially, stock solutions of HA and bovine testicular hyaluronidase were prepared. A stock solution of HA was prepared by dissolving 0.2 g of HA in 50 mL of 0.01 M ammonium acetate buffer to give a final concentration of 4 mg/mL. The final pH

of the solution was 6.5. A stock solution of hyaluronidase was prepared at a concentration of 0.020 g/mL. Reactions were prepared by aliquoting 1 mL of the stock HA solution into 4 microcentrifuge tubes. The protocol by Tawada et al. called for 40 U of hyaluronidase per 4 mg of HA. The enzyme was added at the concentrations of 40, 10, 5 and 2 U. Different enzyme concentrations were used to determine which would give the best range of degradation of the carbohydrate. The microcentrifuge tubes were incubated at 37°C for one hour and boiled to stop the reaction. Characterization of the depolymerized HA was performed by Carmen Batchelor.

3. Results and Discussion

3.A. Assay Development: Detection of CD44 Binding of HA

Several experiments were conducted to optimize conditions for the cell based competition assay. Results of the following experiments are described in the sections as referenced below.

Biotinylated HA was characterized as per section 3.A.1. A precipitation reaction was performed to confirm that HA was present in the samples. Avidin-fluorescein was then added to confirm that the HA was biotinylated. Fluorescence was analyzed using a microtiter plate reader.

In section 3.A.2 we describe the results of an experiment to confirm that Fe was not quenching the fluorophore. If the fluorophore was quenched by Fe, other detection methods would need to be developed.

Section 3.A.3 describes the results of experiments to confirm the presence of the CD44 receptor on the surface of the cells. A primary antibody to CD44 and a secondary antibody conjugated to a fluorophore were incubated with the mesothelioma cells. The presence of a fluorescence signal would indicate that both antibodies were bound to their antigens, thus confirming the presence of CD44.

Section 3.A.4 describes the results of an experiment conducted to confirm that HA was recognized by the CD44 receptor despite biotinylation of HA. An

anti-CD44 primary antibody was used to block the receptor from biotinylated HA. A decrease in fluorescence was observed between the wells in which only biotinylated HA was added and those wells in which primary antibody was added in addition to biotinylated HA. The results indictated that HA was bound to cells via CD44.

Section 3.A.5 describes an experiment demonstrating that biotinylated HA that was bound to CD44 could associate with avidin-fluorescein, and that fluorescence of the complex was detectable. A depletion assay with live cells compared the fluorescence signal of avidin-fluorescein after incubation with biotinylated HA-bound cells to the fluorescence signal of the cells. The cells displayed an increase in fluorescence while the avidin-fluorescein displayed a decrease in fluorescence. The same experiment was then performed using fixed cells. The results indicated that cell fixing does not interfere with CD44 / HA interactions.

3.A.1. Characterization of Biotinylated HA

Upon addition of ethanol to solutions containing either biotinylated HA or biotinylated HA with avidin-fluorescein, a gelatinous precipitate formed. No such precipitates were observed when ethanol was added to avidin-fluorescein, indicating that the HA was necessary for precipitation. These precipitates were isolated and dissolved in HEPES buffer. The fluorescence intensities of the resulting solutions were measured (Table 4). Fluorescence was not detected in

the wells that contained biotinylated HA alone because no reaction was conducted to link avidin-fluorescein to the biotinylated HA, therefore no fluorophore was present in the wells. The sample that contained biotinylated HA with avidin-fluorescein fluoresced as expected since the fluorophore precipitated with the addition of ethanol because it was linked to the carbohydrate.

The stock solution, at a known concentration of 5 µg/mL avidin-fluorescein was far more concentrated than the avidin-fluorescein present in the sample of biotinylated HA with avidin-fluorescein. Since the avidin-biotin association is assumed to occur with high efficiency, the low level of fluorescence in these samples was likely the result of either relatively low levels of biotin on the HA, or low efficiency of the precipitation. The extent of HA biotinylation is unknown.

The sample that contained avidin-fluorescein but not biotinylated HA did not precipitate and therefore, showed little fluorescence. The fluorescence reported for this sample (Table 4) is likely due to residual avidin-fluorescein present in the wells. The precipitation of the biotinylated HA with avidin-fluorescein coupled with a fluorescence intensity of the dissolved precipitate proves that HA was indeed biotinylated. Additionally, this data proves that HA was not preventing the association between biotin and avidin.

Table 4. Characterization of Biotinylated HA.

	HA*	HA* Av-Fl (ppt.)	Av-Fl (ppt.)	Av-Fl stock (no ppt.) + cntrl
Fluorescence signal (AU)	-12	484	307	53665

*Denotes those samples that have been biotinylated.

3.A.2. Fluorescence Quenching by Fe (III) Assay

A decrease in the fluorescence intensity was observed as the concentration of biotinylated Fe complexed HA was increased in the presence of solutions of avidin-fluoresein. However, the fluorescence intensity for all of the samples was higher than that of the control. It has been previously established that an increase in viscosity of a sample and the binding of a fluorophore to a large molecule increases the quantum yield and thus increases fluorescence intensity[17]. We attribute the increase in fluorescence to be due to the high viscosity of our HA solutions and to the presence of high molecular weight HA, which is bound to fluorescein. Furthermore, not all of the biotinylated Fe complexed HA will bind to fluorescein. The presence of free biotinylated Fe complexed HA in the sample is proposed to contribute to additional quenching. In the absence of free Fe complexed HA, we would expect higher fluorescence intensities.

Table 5. Fluorescence Quenching.

Sample	Fluorescence (AU)
Positive Control	57
40 µl HA*-Fe / 0 µl HA*	67
20 µl HA*-Fe / 20 µl HA*	88
10 µl HA*-Fe / 30 µl HA*	100
5 µl HA*-Fe / 45 µl HA*	96

3.A.3. Confirmation of CD44 Expression Using 1° and 2° Antibodies

Results of histochemical staining experiments indicate that CD44 receptors are being expressed by the mesothelioma cells and that the CD44 receptors bind HA and Fe-HA. In this experiment, the cells were treated with anti-CD44 antibodies followed by fluorescently labeled secondary antibodies. The secondary antibodies bound to the Fc region of the primary antibodies, indicating the presence of the CD44 receptors. Furthermore, the fluorescence signal increased when the concentration of secondary antibody increased. From this evidence we conclude that the cell seeding protocol was effective. To prevent damage to the receptors, cell scrapers were used to detach the cells from the cell culture substrate instead of trypsin digestion. We found that a 24 hour incubation was sufficient for the cells to re-express the receptors damaged during the seeding process.

Table 6. Histochemical Confirmation of CD44 Expression.

	1° antibody + 2° Antibody (1:100)	1° antibody + 2° Antibody (1:10)
Fluorescence (AU)	97	100

3.A.4. Confirmation of CD44 Expression by 1° Antibody Blocking

To confirm that CD44 on the cells is the species responsible for binding HA, we measured the effect that treatment with anti-CD44 antibody has on HA binding. The reduction in fluorescence signal between wells containing similar reagents indicates that the primary antibody is binding directly to the receptor, preventing the HA from binding. Additionally, the data suggests that the Fe complexed HA binds to CD44 with lower affinity than the HA since the wells that contain Fe complexed HA have much lower fluorescence intensities.

Table 7. Confirmation of CD44 Expression by Antibody Blocking.

	HA-Fe*	HA*	1° antibody + HA-Fe*	1° antibody + HA*
Fluorescence (AU)	46	100	4	45

* Denotes those samples that have been biotinylated.

3.A.5. Depletion Assay with Live Cells

We conducted depletion assays to evaluate the CD44 / biotinylated HA interaction using avidin-fluorescein binding. Cells were treated with biotinylated HA followed by aliquots of a stock avidin-fluorescein solution, and the amount of avidin-fluorescein removed from the stock solution was determined. Residual avidin-fluorescein (determined in control experiments) was not sufficient to give false positive results. The fluorescence intensity of the positive samples was about 7 times that of the control background. This is concluded to be high enough above background to be detectable in future assays. The well containing the transferred avidin-fluorescein shows a 6% depletion in the fluorescence

signal when compared to the well containing the avidin-fluorescein stock solution. The signal from the well containing the biotinylated HA with avidin-fluorescein is 4% that of the well containing the avidin-fluorescein stock solution. Furthermore, the fluorescence data from the well containing the biotinylated HA with avidin-fluorescein and the well containing the transferred avidin-fluorescein sum to 98%, suggesting that avidin-fluorescein is binding to biotinylated HA which is in turn bound to the CD44 receptor. A 2% loss can be contributed to residual avidin-fluorescein remaining in the well after the contents were transferred and the well was washed.

Table 8. Depletion Assay with Live Cells.

	HA* + Av-Fl + cells	Av-Fl removed + cells	Av-Fl from well 2	Av-Fl Stock Soln.
Fluorescence	4	0.6	94	100

* Denotes those samples that have been biotinylated.

3.A.6. Depletion Assay with Fixed Cells

Results of depletion assays with fixed cells were similar to those obtained from the depletion assay with live cells (Table 9). This suggests that future assays can be conducted using a step that fixes the cells with paraformaldehyde without jeopardizing the results. All future assays will be conducted in such a manner that cells are fixed prior to incubation with avidin-fluorescein.

Table 9. Depletion Assay with Fixed Cells.

	HA* + Av-Fl + cells	Av-Fl removed + cells	Av-Fl from well 2	Av-Fl Stock Soln.
Fluorescence (AU)	3	1	94	100

* Denotes those samples that have been biotinylated.

3.A.7. Competition Assay

When analyzing the wells in which biotinylated Fe complexed HA was added alone, we expected to see values similar to those obtained in the fluorescence quenching experiment for the sample containing 100% Fe complexed HA (section 3.A.2). The results show a slight reduction in the fluorescence intensity for the competition assay, which suggests that CD44 receptors are not being saturated with the biotinylated Fe complexed HA.

Additionally, comparison of the relative fluorescence of the wells treated with Fe complexed HA (49) to those treated with HA (92) shows a decrease in fluorescence intensity for the iron treated samples. We also note a decrease in the relative fluorescence for the incubated samples (61 and 88) when compared to the samples added in tandem (71 and 100). Interestingly, the relative fluorescence of all the samples containing both Fe complexed HA and HA (61 – 100) were significantly greater than those of samples containing Fe complexed HA alone (49). This suggests that some crosslinking between the polyanionic HA chains was occurring, leading to a greater amount of carbohydrate binding per CD44 receptor. We hypothesize that region of the crosslinked polysaccharide that binds to the receptor is the region with the lowest Fe content.

Table 10. Competition Assay.

Labeled Material *	**Single material added**	**Materials incubated together**	**Materials added simultaneously**
HA	92	88	100
Fe-HA	49	61	71

* Hyaluronic acid samples with or without iron were labeled with biotin. Experimental conditions were as described in Section 2.D.5.

4. Conclusion

4.A. Assay Development: Optimized Protocol

4.A.1. Optimization of Cell Culture

The assay development process led to an optimized protocol for use with human mesothelioma cells and biotinylated HA. Experimentally, we determined that trypsinization of the cells resulted in a possible loss of CD44 receptors from the cell surface. As a result, cell scraping was used for gentle removal of the cells from the culture substrate. However, scraping resulted in cell clumping which gave rise to uneven growth on the culture dish. Future assays will be conducted to determine the duration of incubation necessary after trypsinization that allows for re-expression of the CD44 cell surface receptor.

Cell based assays showed that high cell confluence gave rise to a decrease in fluorescence intensity. Cell confluence at 20-30%, observed after a 24 hour incubation period, resulted in the highest fluorescence intensities. When confluence was low, the exposed cell surface area was high, and thus more receptors were available to bind to target molecules.

4.A.2. Optimization of Cell-Based Assays

Adequate well washings after incubation with reagents was necessary for removal of residual reagents. We determined that washing wells for 3 minutes in

PBS (x3) gave acceptably low background fluorescence. Additionally, we experimentally showed that results did not vary between those cells that were fixed and those that were not. Specifically, cell fixing maintained the CD44-biotinylated HA association while not interfering with subsequent avidin-fluorescein binding. These results validated the use of cell fixation in long assays for which cell viability could be problematic.

4.A.3. Optimization of HA Binding

Although CD44 receptors are known for their affinity for HA, it was not previously known if modification of HA would affect that affinity. We discovered that biotinylation of HA did not affect the association. This confirmed that avidin-biotin association could be exploited to detect HA and HA-Fe binding to CD44.

4.B. Competition Assay

Competition assays were conducted to determine the effects of iron(III) on HA binding by CD44 receptors (Sections 2.D.5 and 3.A.7). CD44 receptors expressed by human mesothelioma cells were shown to bind both biotinylated HA and biotinylated FE complexed HA. Iron was shown to partially quench fluorescence of the biotinylated Fe complexed HA avidin-fluorescein conjugate, complicating interpretation of the results. Combining the results of the fluorescence quenching experiments with the biotinylated HA and biotinylated Fe complexed HA experiments, it appears that iron slightly inhibits HA binding.

Specifically, when corrected for quenching effects, the fluorescence observed with biotinylated Fe complexed HA binding experiments is still significantly lower than that observed with biotinylated HA.

While biotinylated HA binding was partially blocked by pre-treatment of the cells with an anti-CD44 antibody, such treatment fully blocked binding of biotinylated Fe complexed HA. This result suggests that biotinylated Fe complexed HA may be binding to a subset of CD44 receptors that are blocked by the antibody, while the biotinylated HA is bound by a larger set of receptors.

Polyanionic HA molecules have been shown to bind metal cations, presumably causing changes in conformation, and possibly crosslinking the normally linear HA chains[15, 31]. Results of these assays were not consistent with a simple competitive binding model (i.e., a model where either HA or Fe complexed HA is bound to a given receptor). While added Fe complexed HA did not compete to diminish biotinylated HA binding, added HA served to increase biotinylated Fe complexed HA binding. This result suggests that incubation of Fe complexed HA with biotinylated HA or incubation of HA with biotinylated Fe complexed HA results in crosslinking of the HA molecules. In this scenario, both HA and biotinylated HA are crosslinked by iron and the resulting complex is anchored to the receptor. Since both samples are attached, additional fluorescence is observed in the case of the biotinylated Fe complexed HA binding.

4.C. Future Experimentation

Future experimentation will seek to confirm quenching of the fluorophore by iron. If quenching is confirmed, other methods of detection will be employed. Additionally, the concentration of HA necessary to saturate the receptors will need to be elucidated. Titration experiments with biotinylated HA can provide this information.

Long term experiments will aim to confirm the formation of a crosslinked complex. Incubation of biotinylated samples of HA and Fe complexed HA would give rise to an increase in fluorescence signal if a crosslinking reaction is occurring. Additionally, depolymerized Fe complexed HA samples will pass through a 0.45 μm microspin column if no crosslinking is occurring. Conversely, crosslinked samples will not pass through the same filter. An experiment will be conducted in which incubated depolymerized Fe complexed HA is centrifuged in a microspin column. Confirmation of crosslinking will be made using EI-MS.

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