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Monitoring the Effects of MMP Inhibitors on Extracellular Matrix Degradation for use in Implant Protection

Jake D. Jones
University of Arkansas, Fayetteville

Monitoring the Effects of MMP Inhibitors on Extracellular Matrix Degradation for use in Implant Protection

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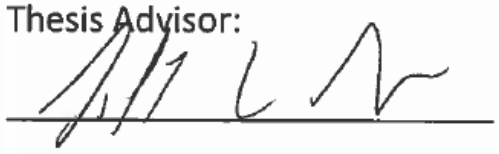
Department of Biomedical Engineering
College of Engineering
University of Arkansas
Fayetteville, AR

By

Jake D. Jones

This thesis is approved.

Thesis Advisor:



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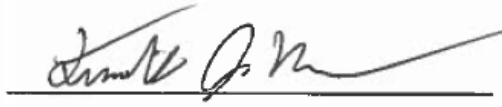

1. Introduction

Over the past few years, the ability to readily harvest the extracellular matrix (ECM) of cells has experienced many innovative developments that have led to an increase in the availability of ECM for study and experimentation. This accessibility along with the long standing history of the ECM as a potential biomaterial has turned into a growing movement to utilize and apply the ECM and its byproducts to biomedical research and therapies [1]. A breadth of applications has emerged spanning uses such as three-dimensional scaffolds in cell culture research to direct medical procedures implemented as fillers for ostechondral defects and the creation of cardiac and vasculature reconstructive tissue for implantation [2]. The adaptable nature of the ECM along with its favored properties of biodegradation and biocompatibility make it highly desirable as an implantable biomaterial, but problems have arisen during its implementation that have hampered its therapeutic potential. In particular, one of the greatest benefits with of ECM-based implantations may also be one of the greatest limiting factors –the short time in which it is biodegraded or resorbed [3]. As the body has the ability to produce ECM, so it too possesses the ability to resorb it which it readily does when large quantities of implanted ECM are introduced *in vivo*. A pharmaceutical or chemical treatment to improve the functional time of implantable ECM is potentially available, but there exists a need for a cost effective, *in vitro* model to evaluate these inhibitory agents for their ability to hamper ECM degradation.

1.1 The Physiological Environment of Extracellular Matrix

The extracellular matrix is not definable by its material alone as its composition can differ depending on the microenvironment of the body or organism that houses it. Instead, the ECM is most definable by its functional characteristics including its abilities to allow cellular adhesion, provide structure, and even act as a repertoire for intercellular signals such as growth factors [3]. That being said, all ECM shares some common compositional components with the most abundant of these being the structural proteins- collagen and elastin- and the adhesive proteins- fibrinogen and laminin [4]. In particular, collagen is the most common underlying protein found in ECM comprising up to 90% of the dry weight in some tissues [5]. Collagen is subdivided into twenty-eight types to better distinguish among its structures and functions. The most widespread of these functioning in the ECM are types I and III [5] utilized in a span of biological locations ranging from structure of skin, vasculature, interstitial tissue, granulation tissue and scar tissue [6]. Due to this prevalence throughout the body, collagen is one of the major targets of reconstruction since it comprises most of the structure of the ECM. It was for this reason that the model system to evaluate ECM degradation used DQ collagen as an analog. DQ collagen is a collagen conjugate molecule that susceptible to all the same enzymes of natural collagen (Figure 1). It is conjugated with a fluorescent molecule that is only exposed when the DQ collagen is cleaved, allowing variations in the level of collagen degradation to be evaluated by measuring changes in expressed fluorescence [7]. The molecules that cause this degradation are naturally occurring proteins called matrix metalloproteases (MMP) which can be found anywhere there is ECM reconstruction.

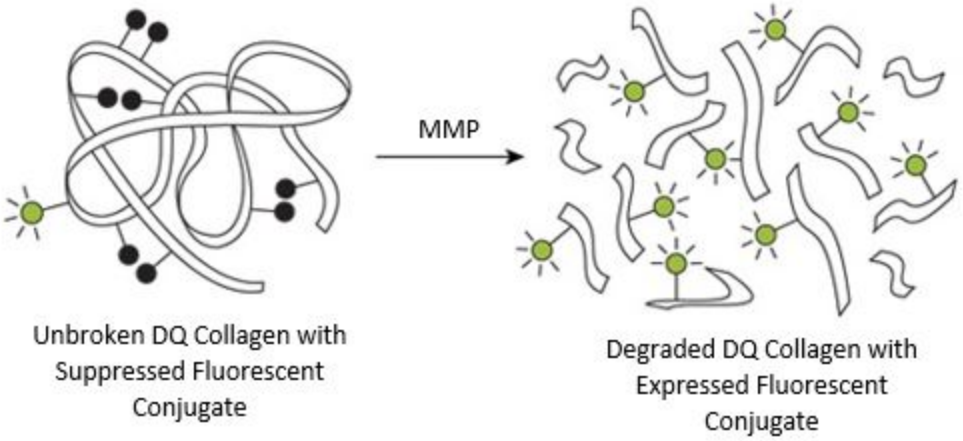

Figure 1. Representation of DQ collagen structure before and after degradation by MMPs. Before degradation, fluorescent signal is quenched by close proximity of fluorescent molecules on the intact collagen [7]. As MMPs degrade the collagen structure, more fluorescent molecules are exposed resulting in a greater signal. Ownership of graphic belongs to http://aetherforce.com/how-fluorescent-light-works/.

These MMPs operate with the use of a catalytic Zn^{2+} active site in their structure allowing the hydrolysis of ECM proteins [8]. The MMP group that specifically degrades collagen is known as the collagenases. The collagenases operate as endopeptidases cleaving collagens I, II, and III at an interior-domain site marked by the N-terminus [8]. Due to the effectiveness of MMPs at low concentrations over short time intervals, they are synthesized as zymogens that must have a section of the pro-peptide domain removed in order for the zinc-dependent active site to become available [8]. In addition, members of another family of proteins known as the tissue inhibitors of metalloproteinase (TIMPs) are produced in areas of reconstruction to keep MMP activity in check. Physiological conditions of reconstruction that lead to matrix metalloproteases synthesis are angiogenesis, wound healing, and tissue remodeling for growth or normal wear.

The cells responsible for ECM reconstruction are known as fibroblasts. These cells produce and export both the proteins that comprise the ECM as well as the MMPs that help remove them. To accommodate this high level of protein secretion, fibroblasts are characterized by extensive endoplasmic reticulum and long, branched cytoplasm. Their primary goal is to

maintain the structure of the ECM and as such they are found in connective tissues and in sites of wound healing. In the model of ECM degradation, 3T3 cells were used as analogs for a potential reconstructive environment. The 3T3 cells are fibroblasts capable of both ECM secretion and the ability to secrete collagen degrading MMPs that are necessary to break down the structure of DQ collagen [9].

1.2 Potential Control Mechanisms for Degradation of Extracellular Matrix Implants

In order to lengthen the functional time of implantable ECM, the mechanism of degradation must be inhibited. The matrix metalloproteases are the primary method by which ECM is broken down, and the TIMP family of proteins exists as proof that inhibition of these proteases is possible naturally. In particular, the glycoprotein TIMP-1 plays an inhibitory role with most of the MMPs including the collagenases [10]. Furthermore, there exist synthetic or chemical molecules that likewise inhibit the catalytic actions of MMPs. One such molecule is Batimastat (BB-94) which has been proven to be a dose dependent inhibitor of MMPs during the inflammatory response [11]. In order to evaluate the potential to increase the effective time of ECM based implants, an *in vitro* assay was developed to monitor the natural degradation rate of ECM in culture with DQ collagen and 3T3 fibroblasts. The protein TIMP-1 and the molecule BB-94 were incorporated into this model to evaluate their potential use in controlling the degradation rate of ECM implants.

2. Methods

2.1 Preparation of Foam Scaffold and Well Plates

Foam scaffolds were prepared by first creating a polymer solution of polyurethane and DMAC. The solution was prepared with 2g of polyurethane beads being allowed to dissolve completely in 20 mL of DMAC over a 24 hour period. The next step involved mixing 10 grams of table sugar with 200 μL of deionized water to create a sugar slurry. The sugar slurry was spread into molds -dimension 15 mm diameter by 1 mm height- sized for use in twenty-four well plates. The molds were then placed into an oven at 60 degrees centigrade for 20 minutes to allow some of the excess water to evaporate off. At this point the polymer solution was applied to sugar filled molds until the molds were filled. This required between an average of 1.2 to 1.4 mL of polymer solution per well. The polyurethane foams were then submerged in deionized water for 24 hours at room temperature. Following the first 24 hour period, the now solid and porous polyurethane foams were removed from their molds and placed in a separate DI water bath at room temperature. The new bath was stirred constantly, and the DI water was changed every 8 hours for another 24 hour period to remove all traces of remaining sugar. The foams were then removed from the bath and placed 8 apiece into 50 mL centrifuge tubes and allowed to freeze overnight in a -25 degree centigrade freezer. After the foams were completely frozen, they were lyophilized for another 24 hours. The foams were stored sealed at room temperature until placed into a twenty-four well plate and sterilized using gas sterilization.

2.2 Cell Culture

Frozen vials of 3T3 fibroblasts were thawed in 37 C water bath and added to 16 mL of warm media at same temperature in a T-175 flask. Following seeding, cells were incubated for 45 minutes at 37 C. The media was then removed and replaced so only the cells that adhered to the flask would remain. Cells were incubated at 37 C. Cells were fed every 24 hours with a fresh 17 mL of media until confluence. Average times of confluence ranged between 5-7 days.

Following confluence, cells were then split into three flasks. After removing all media from the flask, trypsin EDTA (7 mL) was added and allowed to incubate with the cells for 15 minutes. After this time, 7 mL of warm media were added to neutralize the trypsin. The resulting 14 mL solution was put into a centrifuge vial and spun down at 300 G for 5 minutes. The remaining solution was removed and the resulting pellet was re-suspended in 12 mL of media. This new solution was added to 3 T-175 flasks (4 mL each) along with 13 mL warm media. These flasks were cultured using the above steps until confluence.

2.3 Well Plate Preparation

Foams that had been gas sterilized were treated with fibronectin before cells were seeded onto them. To start, the sterile foams were rinsed with 500 μL of sterile PBS and left to sit for 5 minutes. The foams were each rinsed a total of three times. Fibronectin solution (500 μL) was then applied to the foams. Well plates were wrapped in para-film and stored cold for 36 hours.

Cells were removed from T-175 flasks using steps described in 2.2. Following removal, the cells were counted using a hemocytometer. A total of 8 million cells were seeded per foam and warm media was added until the total solution in each individual well was 500 μL. The well plates were then incubated for 24 hours at 37 C.

2.4 Testing for Signal to Noise

Two well plates were used for this test. One well plate had half of its wells left without foams in order to establish controls on the degradation rate of the DQ collagen. The DQ collagen was prepared in three concentrations of 2 μg/mL, 10 μg/mL, and 50 μg/mL. A total of nine foams would be exposed to each concentration over a period of 4 days. The first three foams from each concentration (n=3) would be sampled after 24 hours, the next three foams would be sampled after 48 hours, and the final 3 foams would be sampled at 96 hours along with each of the foamless control wells. To sample the wells, three 100 μL samples were taken from the 500 μL solution in each well. The 100 μL samples were put into a 96 well plate. Fluorescent readings were taken on days 1, 2 and 4 with excitation wavelengths at 485/20 nm and emission wavelengths at 528/20 nm to best comply with manufacturer's suggestions. Foams were removed from original plate at time of sampling and placed into a new sterile well plate before being suspended in 10% formalin. DAPI staining was performed on foams to make sure cell adhesion had taken place and the well samples were valid (Figure 2). Test was repeated twice.

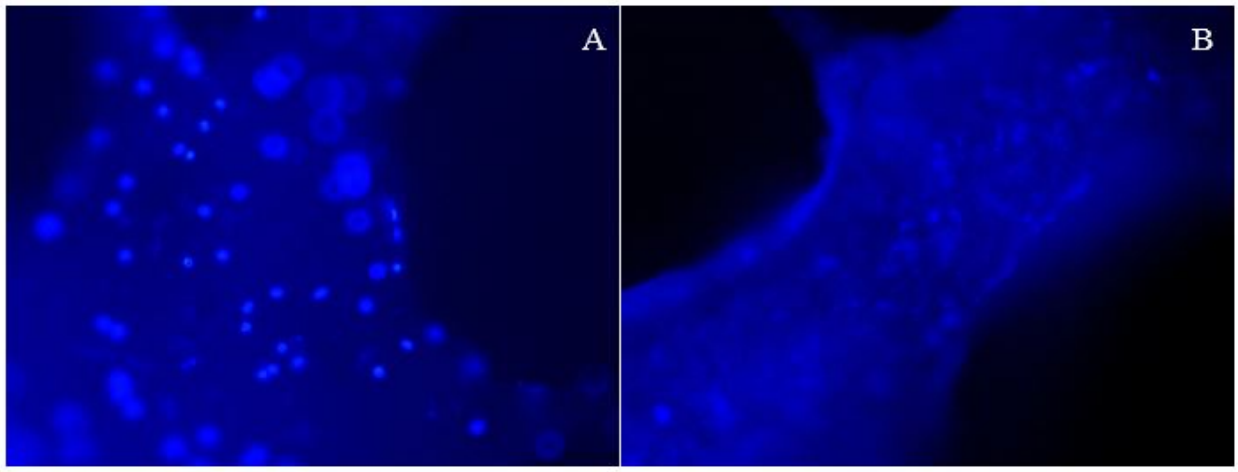

Figure 2. Representation of DAPI staining results from the seeded foam scaffoldings that had participated in the assay. Foam A is an example of a foam that was successfully seeded as seen by the prevalence of distinguishable nuclei visible on the polyurethane foam. Foam B is an example of a foam that was eliminated from the assay for failure to seed any cells.

2.5 Effect of TIMP-1 and BB-94 on MMP Inhibition

Similar to the signal to noise test, two 24 well plates were used for this test. All foams in this test received DQ collagen at the concentration and for the amount of time that yielded the greatest signal to noise ratio in the previous tests. TIMP-1 working solution was prepared with sterile PBS to form concentrations of 2 ng/mL, 10 ng/mL, and 100 ng/mL when added to the 500 μL of media in each well. Batimistat was made into working solution with DMSO at concentrations of 10 nM, 100 nM, and 1000 nM when added to the 500 μL of media in each well. BB-94 solution was added so that the total amount of solution in the well was less than 5% DMSO do minimize its effects on the cells. The DMSO was also sterile filtered to minimize chance of contamination.

One well plate was filled with seeded foams. Each column corresponded to one of the concentrations of BB-94 or TIMP-1 listed above (n=4). Only eight foams were added to the other plate which received no MMP inhibitor. Samples were taken in the same manner as in 2.4. Following the sample collection phase, foams were fixed in 10% formalin in a new well plate. Foams were DAPI stained to check for cellular adhesion to the foam. Sample foams without cell adhesion were eliminated from being represented in the final results.

2.6 Statistical Analysis

All data sets are represented by the mean and standard deviation. Comparisons between samples groups were evaluated using a student's t-test. A standard 0.05 level of significance was used for all statistical tests.

3. Results

The first phase of experimentation was performed in order to find an acceptable signal to noise ratio of testing over a period of four days. Readings were taken on days 1, 2, and 4. The first run had two samples that performed differently than the controls at the level of statistical significance (Figure 3). The first occurred at day 2 at a concentration of 50 ug/mL. The mean value of the sample was 1612 ± 18.76 while the mean for the control was 1409 ± 16.08 giving < 1% chance that the two groups were the same. The second notable sample occurred on day 4 at the concentration of 50 ug/mL DQ collagen. The mean value of the sample was 2292 ± 102.4 with the mean of the control being 1427 ±

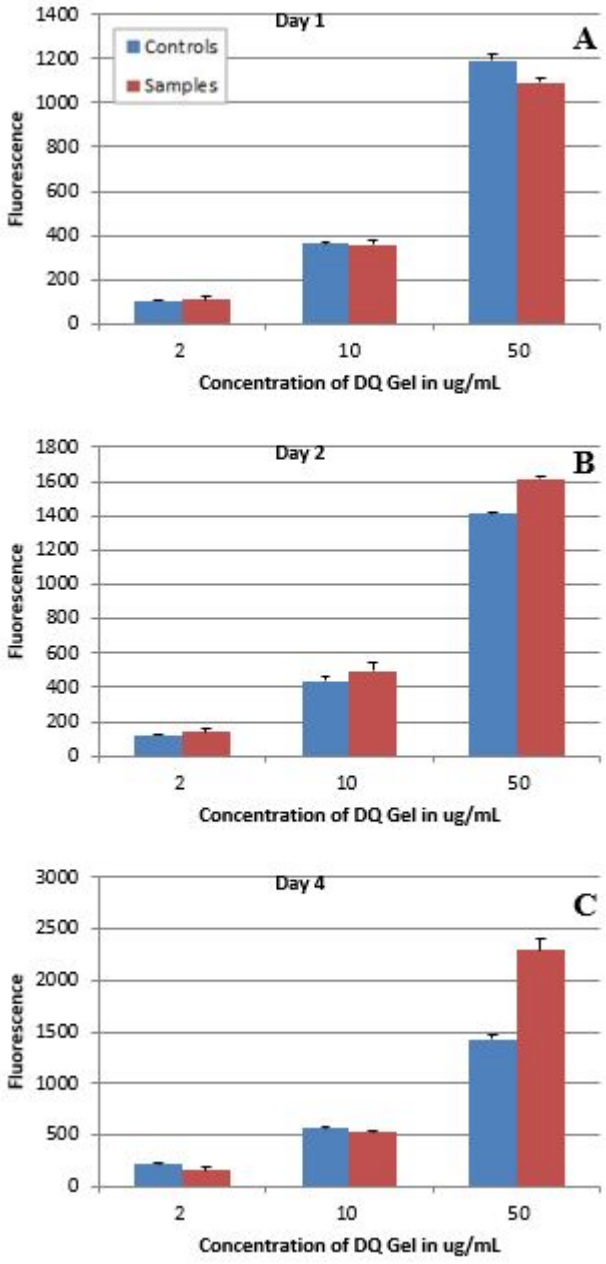

Figure 3. Fluorescent values for concentration of DQ collagen in presence of fibroblasts, first run. Only 50 ug/mL in B and C were shown to be statistically significant with signal to noise ratios of about 8:7 and 2:1 respectively.

50.76 also giving < 1% probability that the two groups were the same.

The second run of the assay likewise resulted in two samples that displayed significantly different fluorescent values. However, both of these samples appeared only on the fourth day of evaluation (Figure 4). The first was the 2 ug/mL concentration of DQ collagen at an average of 412 ± 23.96 for the sample and an average of 277.7 ± 3.858 giving < 1% chance that the sample and control were the same. The second was at the 50 ug/mL concentration at an average of 2717 ± 233.8 for the sample with the control averaging at 2048 ± 31.51 yielding a probability of < 1% that the two groups were the same. From the above assays it was decided that the most substantial signal to noise ratio existed at the fourth day at 50 ug/mL of DQ collagen since both assays displayed statistical significance at these points.

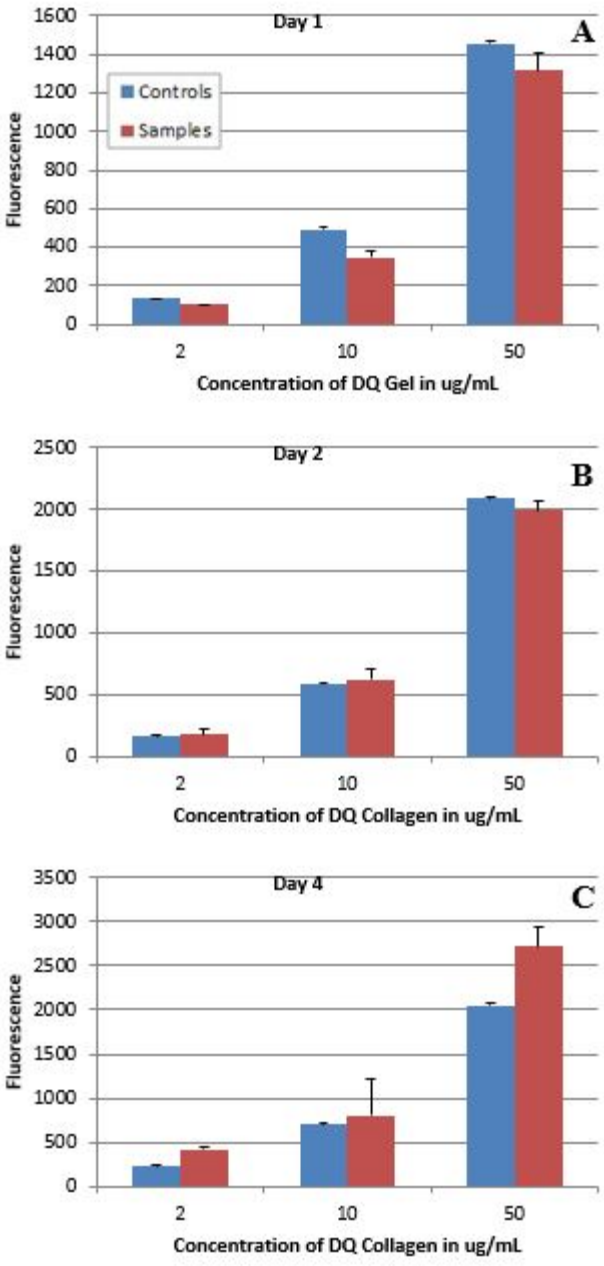

Figure 4. Fluorescent values for concentration of DQ collagen in presence of fibroblasts, second run. Two samples in C at 2 and 50 ug/mL were shown to be statistically significant with signal to noise ratios of about 4:3 and 7:5 respectively.

The final test was used to determine the effectiveness of BB-94 and TIMP-1 to prevent collagen degradation (Figure 5). From the two MMP inhibitors, there were a total of three concentrations that were shown to be

statistically less degraded than the controls. The first was the only concentration of TIMP-1 that was shown to be potentially significant, and it occurred at the lowest concentration of only 1 ng/mL. The mean of this TIMP-1 sample was 1878 ± 62.56 as compared to the controls mean of 2252 ± 175.9 giving a probability value of < 5%. Batimistat was shown to reduce degradation at concentrations of 100 nM and 1000 nM. The mean fluorescent value at 100 nM was 1832 ± 109.2 while the mean value for 1000 nM was 1751.5 ± 158.6 showing that both had < 5% probability of being from the control group.

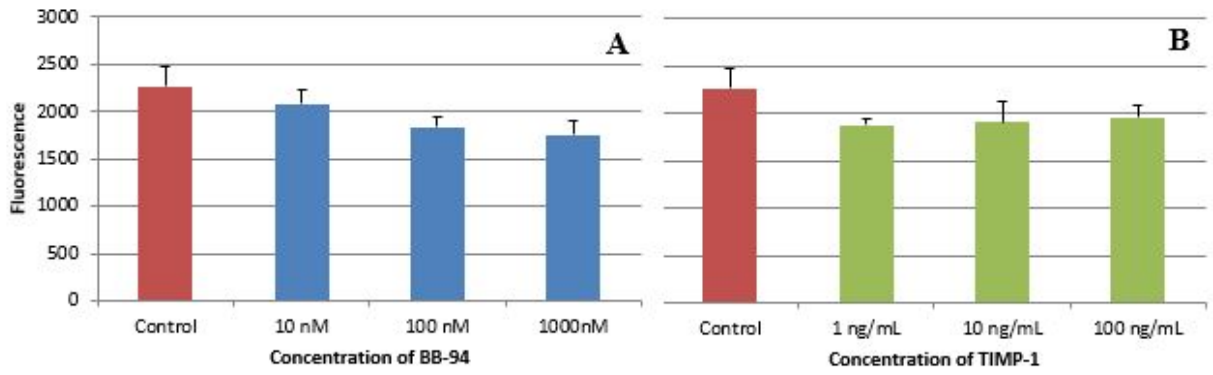

Figure 5. Results of assay testing with inhibitory agents BB-94 (A) and TIMP-1 (B). BB-94 showed significant results at 100 nm and 1000 nm with means of 1832 ± 109.2 and 1751.5 ± 158.6 respectively. TIMP-1 only was shown to be successful in MMP inhibition at 1 ng/mL concentration with a mean of 1878 ± 62.56. The control group represented had a mean of 2252 ± 175.9, which is higher than in either of the previous assays.

4. Discussion

The signal to noise ratio collected from the first two assays was most acceptable for the time period of 4 days at a concentration of DQ collagen at 50 ug/mL. Both assays showed that this result was statistically significant from the control populations, and was visibly more distinguishable when plotted due to a larger difference in the mean fluorescent values. A possible explanation for these results is the higher concentration of DQ collagen was likely more sensitive to the MMPs secreted by the 3T3 cells since the chances of an MMP finding a binding

site to initiate cleavage were greater. The 4 day time period allowed the cells to be metabolically active longer thereby increasing the concentration of functional MMPs in the model.

When the inhibitory molecule BB-94 and the MMP inhibitor protein TIMP-1 were introduced, the results displayed distinctive patterns of success. The assay displayed a pattern of increasing inhibition effectiveness with an increase in concentration of BB-94. Batimistat was successful in the inhibition of MMPs at concentrations of both 100 nM and 1000 nM. Although these two groups were independent of the control group, they were statistically similar potentially implying that the most efficient concentration of BB-94 lies at or around 100 nM with diminishing returns resulting from introducing higher concentrations of the drug. However, more testing would be needed to support this hypothesis. TIMP-1 yielded surprising results with only the lowest concentration being statistically successful at inhibition when compared to the controls. A caveat to this result is that the populations of TIMP-1 concentrations were statistically indistinguishable from one another with a much more comparable mean than the BB-94 populations. The results also do not indicate that increasing the concentration of TIMP-1 will have any effect on the inhibition of MMPs within the scope of concentrations tested.

This particular run of the assay was limited by the inclusion of only fibroblasts within the model. When dealing with ECM reconstruction *in vivo*, there are other cell types that would contribute to the body's response to an implant, namely macrophages and endothelial cells. Macrophages are common responders to all medical implants *in vivo* since they are mediators of the inflammatory response to foreign material [12]. Endothelial cells are found throughout the body in various forms, but mainly serve to regulate vasculature systems through protein expression including the excretion of MMPs during reconstructive events such as angiogenesis [13]. Although the model did not include these cells initially, it is easily adaptable to

accommodate them in future iterations since the structural foam for cell seeding is coated in fibronectin, a glycoprotein known to attach both endothelial cells and macrophages [14]. Other inhibitory molecules or proteins can likewise be readily incorporated into the assay since the setup in not inherently dependent on the choice of inhibitor molecule. Further testing could also be done employing therapeutic MMP inhibitors already approved by the FDA such as Periostat [15] or developing therapeutics such as the breast cancer treatment drug Tanomastat [16]. Overall, the assay proved to be successful in its ability to determine the effectiveness of MMP inhibitors as potential protectors to ECM based biomaterials by showing the inhibitory effects of BB-94 and TIMP-1. Furthermore, it displays a degree of versatility that will allow it to be utilized in successive evaluations of inhibitory molecules and proteins.

5. Acknowledgements/References

5.1 Acknowledgements

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