Development of a Blood Brain Barrier (BBB) Mimetic to Study Breast-Brain Metastasis

THESIS

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Abstract

Cancer metastasis is a highly complex process that causes 90% of all solid tumor deaths.
[1] With recent advances in diagnostic modalities and treatment options, the occurrence of
brain metastasis has been rising over the last decade.[2] Since many therapeutics are unable
to cross the blood brain barrier (BBB), a protective layer separating the vascular system
and the brain, brain metastases are notoriously difficult to treat. In certain types of cancer,
tumor cells can invade the brain by crossing the BBB from the circulatory system through
a process known as extravasation. Brain metastasis in breast cancer leads to poor
prognosis with mean survival rate of 2 years,[3] Studying the mechanism of the
extravasation of breast cancer into the brain is critical for the elucidation of the pathways
driving this metastatic process. Current methods used to study this invasion process
cannot fully recapitulate physiological conditions. The gold standard method uses
Transwell® inserts that have a non-physiological membrane separating the 'blood' and
the 'brain stroma', which can cause non-physiological behaviors in migration studies. [4]
Thus, we developed a three dimensional (3D) 3-layer hydrogel model to study the
invasion of breast cancer into the brain. To develop this model, the physical effects of
composite Hyaluronic acid (HA) / collagen matrices used as brain stroma mimetics in
breast-brain metastasis were investigated. HA was chosen because it is one of the most
common glycosaminoglycans found in the brain extracellular matrix (ECM) [5] and
collagen was chosen because it is a major component of the basement membrane of the

BBB.[6] In this study, highly invasive MDA-MB231 breast cancer cells were either encapsulated in or suspended on the surface of the composite hydrogels and the migration velocity was ascertained. It was found that cell proliferation was inhibited by HA concentrations higher than 0.5wt%. Adhesion of cells onto the gel surface and cell migration velocity were decreased with increasing concentration of HA in gel composites. Moreover, cell migration velocity appeared to increasing with time (i.e., it is higher on day 5 than on day 1 of the study), potentially indicating remodeling of the ECM by cancer cells or altered chemical signaling from the composite hydrogel matrix. These results suggest that the HA/collagen composite hydrogel is adequate in modelling the brain stroma and further studies optimizing our proposed BBB mimetic are proposed. If successful, this model could lead to better therapeutics that could help hinder or even prevent brain metastases from occurring.

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Chapter 1: Introduction

Metastasis

Metastasis is a complex, multi-step process that involves cancer cell intravasation, circulation in the bloodstream, extravasation, and proliferation at a secondary site. Metastasis accounts for 90% of all deaths resulting from solid tumor cancers and is one of the biggest challenges in cancer treatment. [1] The need to understand the mechanisms of metastasis to improve the patient care is thus urgent and pertinent.

Breast cancer is a predominant form of cancer among women, being the second leading cause of cancer deaths among women in the United States.[7] For breast cancers, there are 4 primary sites for metastasis: namely, the lungs, liver, bones, and brain. In one of the earliest works on breast cancer metastasis, Paget *et. al* observed the spreading of breast cancer to these few organs and suggested that certain organs are predisposed to be prime metastatic sites.[8] The tumor microenvironment, extracellular matrix (ECM) composition, and tissue found at a predisposed metastatic site are very likely to influence the extravasation and growth steps of the metastatic process.

Among the 4 primary metastatic sites for breast cancer, the brain is a metastatic site of interest. Given that brain metastases develops in 10-16% of patients with breast cancer and as many as 30% in autopsies[2] and that patients with brain metastases have a mean survival of 2 years,[3] it is arguably the metastatic site associated with the worst

prognosis. Moreover, the incidence rate of breast to brain metastasis has been increasing over the last decade. This can be attributed to the advancement of medical imaging technologies and systemic treatment options.[2] Thus, a more profound understanding of brain metastasis and its associated mechanisms is absolutely crucial in improving the treatment of breast cancer.

Furthermore, brain metastases often lead to the worst outcomes and present a clinical treatment challenge. Severe neurological complications, like brain herniation and seizures,[9] are likely to occur; and brain tumors show high resistance to standard treatments, such as chemotherapy and radiation.[10] This resistance in systemic treatments is likely conferred by the blood brain barrier (BBB), a protective barrier between the blood and brain, forming an interface that prevents pathogens and most substances from entering the brain.

It should be noted that brain metastasis is a late stage process in breast cancer metastasis.[11] Thus, there is a long latency period before primary breast cancer cells are transformed into cells that are viable in the brain ECM. This implies that breast cancer cells cannot initially invade the BBB or proliferate in the brain microenvironment, but can eventually do so under selective pressure from the environment. Therefore, it is important to study how brain ECM affects breast cancer cell migration, as the ECM is likely to influence the metastasis process.

Blood Brain Barrier

The BBB is a physical barrier between the blood vessels and the brain, comprised of capillaries with tight junctions and no fenestra. The tight junctions hence confer it a low permeability to solutes, a high electrical resistance and regulates the flow of ions, nutrients and protects the brain against pathogens and neurotoxins. They are also linked to the actin cytoskeleton while might allow it to impact the gene expression of the endothelial cells. [12] The proteins found in this tight junction include claudins, occudins and zonula occulin proteins.

The BBB consists of endothelial cells, which form tight junctions that serve as the primary barrier, surrounded by pericytes. Pericytes are involved in the maturation, formation, and regulation of the BBB through signaling pathways that are not fully understood.[13] They are organized in a structure akin to smooth muscle cells in larger blood vessels[14] with endothelial cells in the interior. This endothelial-pericytic complex is then encapsulated by a basement membrane that is ensheathed by astrocytes. The astrocytes thus form another barrier with its tight foot processes, protecting the abluminal side of the barrier. The endothelial cell layer, pericytes and astrocytes would thus form the "neurovascular unit". (Figure 1) [15]

The BBB is also one of the most selective membrane barriers in the human body, allowing nothing but nutrients and small molecules to pass. It protects the brain tissue from pathogens, while allowing homeostatic functions through the regulation of tight gap junctions. Recent studies have shown that the ECM is important in maintaining BBB functions, aiding in the maintenance of barrier proteins. The ECM is also able to reinduce

BBB marker proteins in endothelial cells.[16] Although BBB is a natural barrier that shields the brain from toxins, it also poses a huge challenge for many pharmaceutical drugs to penetrate into the brain. For example, it hinders the delivery of chemotherapeutic agents into the tumor area, and therefore decreases their efficacy.
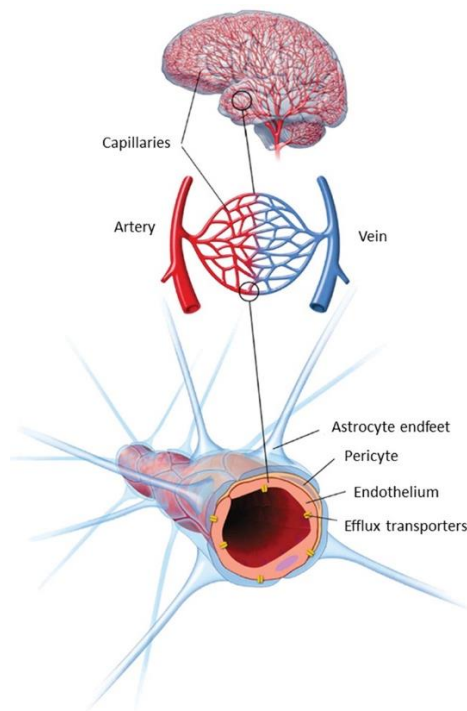
Figure 1: Schematic of a neurovascular unit[15]

In breast to brain metastasis, BBB most likely defends the brain against breast cancer cells initially, but once brain metastases are established, the BBB starts hinders their treatment. It has been proposed that breast to brain metastasis involves the tumor cell adhesion to the endothelium, causing the endothelium to retract, exposing the vascular basement membrane that the tumor cells bind to.[12]

With a rising incidence of breast to brain metastases, it is of clinical relevance to understand the mechanisms of tumor invasion through BBB. The elucidation of cellular

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pathways activated can potentially give rise to new treatments targeting breast-brain

metastases. However, current models that are used to investigate such cancer invasion are

inadequate in creating a close physiological representation of the tumor

microenvironment.

Current Models

Traditionally, two-dimensional models, like the scratch wound assay, and animal

models have been employed in the investigation of metastasis.[17] However, 2D models

often fail to recapitulate the complex tumor microenvironment because of a lack of cell-

matrix communication. It has been shown that the 3D environment regulates the

expression of genes linked to cancer development and metastasis.[18] For instance, gene

expression has been shown to be affected by ECM properties like matrix stiffness[19],

composition[20] and biochemical signals from adjacent or distant cells.[21] With the

dimensionality of the substrate playing a large role in the process of metastasis, 2D

models may no longer adequate to fully understand cancer invasion into the BBB. Thus,

3D models may provide a more physiologically relevant solution to evaluate the forces

driving metastasis.

Clearly, animal models best capture the physiological condition associated with

cancer cell migration; however, animal models are not always feasible because of time

and cost considerations.[22] Moreover, given the complexity of animal models, these

models cannot be easily used to display a specific set of physical or chemical properties

that might be unique to humans.[23] Therefore, *in-vitro* models offer a good complement to

in vivo studies with low cost, repeatability, and providing precise control over environmental cues. [24]

The current gold standard for evaluating cancer cell migration is the Transwell® assay.[25] Transwell® assays have the merit of replicating the 3D environment similarly to *in vivo* conditions, but they employ a porous filter whose membrane pores are blocked by ECM such that non-invading cells are unable to pass.[4] This porous membrane in Transwell® assays is not found in the body and is much stiffer than the native environment, providing non-physiological inputs into the microenvironment.

Thus, we developed a 3D hydrogel model to investigate breast-brain metastasis that does not include membranes. Our model was designed to mimic the BBB microenvironment that breast cancer cells experience in the process of extravasation into the brain. The model is composed of a brain stroma mimetic layer, a BBB mimetic layer, and a breast cancer cell layer (Figure 2).
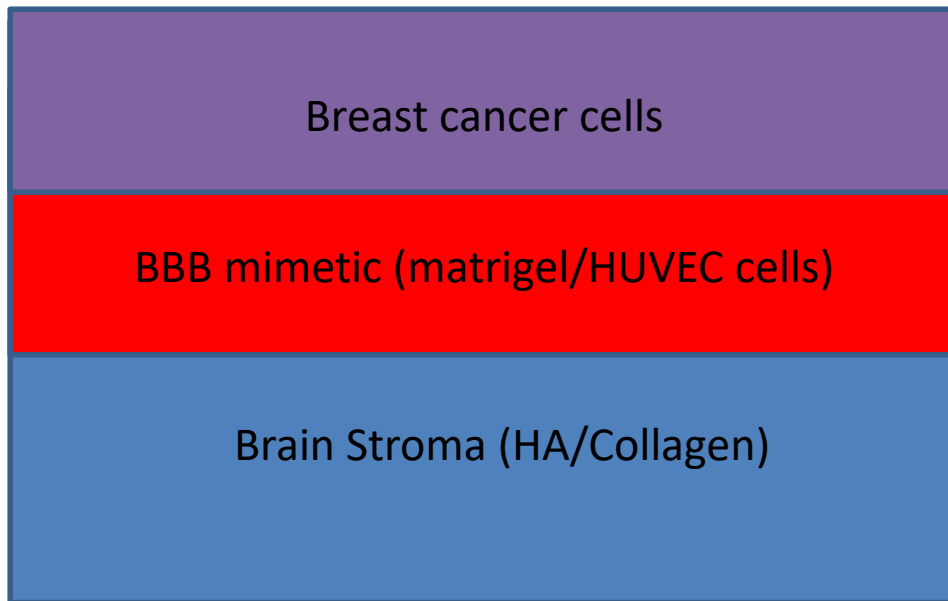
Figure 2: BBB model for analysis of Breast-Brain Metastasis

For the brain stromal mimetic, a composite hyaluronic acid (HA)/collagen hydrogel was used. These were chosen because HA is one of the main ECM components in the brain,[5] and collagen is a primary connective tissue component.[6] Therefore, an HA/Collagen composite hydrogel should display characteristics found in the brain ECM. We chose gel composites over homogenous gels because these increase the complexity of the 3D microenvironment, making it more physiologically relevant.[26]

The BBB mimetics were comprised of human umbilical vein endothelial cells (HUVECs). In native BBB, endothelial cells are characterized by the presence of tight gap junctions and ability to form a monolayer. Thus, this approach provides a BBB layer for the invading breast cancer cells to penetrate and then eventually migrate into the brain stroma, thus, mimicking the extravasation step of the metastatic process.

With migration being affected by both chemical and mechanical cues, it is important to ascertain the influence of physical parameters, like stiffness, porosity, and fiber density of the composite hydrogel in our 3D model before any concrete studies on the chemical pathways are conducted. Thus, in this study, the mechanical effects of the HA/Collagen hydrogel on breast cancer migration were investigated.

Chapter 2: Methods

Cell Culture of MDA-MB231 Cells

Highly invasive MDA-MB231 breast cancer cells were maintained in an

environment at 37˚C and 5% CO_2 and fed every 3 days with media comprised of 89%

Dulbecco's Modified Eagle Medium (DMEM, Invitrogen), 10% fetal bovine serum and

supplemented with 1% penicillin-streptomycin (Invitrogen) and 1X MycoZap (Lonza).

Cells were passaged upon reaching ~80% confluency. To passage, the cells were washed

with sterile phosphate buffered saline (PBS) and detached using 0.25% Trypsin in

Ethylenediaminetetraacetic acid (TRED, Life Technologies) for 5 minutes. TRED was

then quenched with cell culture media, and cells were centrifuged at 3500 rpm for 3

minutes. The resulting cell pellet was then resuspended in 1mL of media and plated onto

new VWR tissue culture dishes at a passage ratio of 1:2.

Preparation of HA/Collagen Composite Hydrogels

Cell Encapsulation in Hydrogels

HA/Collagen composite hydrogels were created using Collagen Type I

(Advanced BioMatrix Inc.) and thiolated hyaluronic acid (HA) (Glycosan Biosystems

Inc.). Both collagen and thiolated HA have the ability to form hydrogels, independent of

one another, at 37° C. Thus, cells can be encapsulated in these hydrogels if added while the hydrogel is setting. 20 mg/mL of thiolated HA was prepared by dissolving the thiolated HA in deionized water. The thiolated HA solution was then mixed with 5 mg/mL solutions of Type I Collagen and Extralink (Advanced Biomatrix) at a ratio of 1:3 Extralink to HA. Extralink is a polyethylene glycol diacrylate that crosslinks with thiolated HA and thus accelerates the HA curing process.[27] This yielded composite hydrogels of titrated HA concentrations (0-1% wt/vol). 100 µL of each composite hydrogel formulation was then added to 48 well plates to create a base layer to prevent cells from settling to the bottom of the well plate. After 1 hour of gel curing at 37˚C in a 5% CO_2 environment, green fluorescent protein (GFP) expressing MDA-MB231 cells in 100 µL of composite hydrogel solution was added to the respective gel base layers to achieve a cell density of ~20 cells/mm^2 and allowed to cure for 1 hour. Thus, hydrogel composites with constant Type I Collagen concentration of 1 mg/mL and HA concentrations ranging from 0 to 10 mg/mL (0-1% wt/vol%) (n=3, see Table 1 for all compositions) were created. Similarly, 100 µL of Matrigel was used as a base and allowed to set for 1 hour before GFP expressing MDA-MB231 cells in 100 µL of Matrigel was added as a control. 1mL of appropriate cell culture media was then added to the wells and allowed to incubate.

Cells Suspended on Hydrogel Surfaces

As previously described, hydrogel composites with constant Type I Collagen concentration of 1 mg/mL and HA concentrations ranging from 0 to 10 mg/mL (0-1%

wt/vol%) and Matrigel hydrogels (n=3, see Table 1 for all compositions) were prepared.

The hydrogels were then allowed to set at 37°C in a 5% CO_2 environment for 2 hours.

Cells were then added to the composites to achieve a final density of ~20 cells/mm^2 while

being supplemented by appropriate media. For the glass control condition, cells were

suspended in media before being added directly to a well in the 48-well plate.

Table 1: Composition of Hydrogels examined

Sample ID (wt%)	HA Control	Col Control	HA 0.1	HA 0.2	HA 0.5	HA 1.0	Matrigel	Glass
Amount of extralink (uL)	16.67	0	1.67	3.33	8.27	16.67	0	0
Amount of HA (uL)	33.33	0	3.33	6.67	16.53	33.33	0	0
Amount of Collagen (uL)	0	20	20	20	20	20	0	0
Amount of Matrigel (uL)	0	0	0	0	0	0	100	0
Amount of Media (uL)	50	80	75	70	55.2	30	0	0

Non-directional Time Lapse Fluorescence Tracking

Cell invasion experiments were performed with an epi-fluorescent microscope (IX71, Olympus), equipped with a motorized stage and an incubation chamber, creating a 37˚C, 5% CO_2 environment. After a 12-hour incubation period in a 37˚C, 5% CO_2 incubator, the invasion of cells was tracked via a series of image collected every 30 minutes for a total of 9 hours. The images were then converted into a movie using the software, Metamorph (Molecular Devices LLC) and processed with the StackReg Plugin for the NIH ImageJ (http://imagej.net/StackReg) software package to correct for the swelling of gels. The cell migration velocity was then calculated via the MTrackJ plugin for ImageJ for individual cells (N≥25 cells for each condition) for each hydrogel sample (N=3 hydrogels). Only cells not undergoing mitosis were considered in the selection of cells for tracking. It should be noted that since the focus is on the effects of ECM on cancer migration, there is no chemotaxic gradient and the migration that we are measuring are non-directional vibrational movement. The migration speeds were then reported in a box and whiskers plot, portraying the mean, median, and outliers for each condition. This was repeated at Day 5 and the migration velocity of the MDA-MB231 cells was similarly analyzed for 9 hours at 30 minute intervals.

Statistical Analysis

Statistical Analysis was performed using JMP software and a Tukey's range test was then performed at a 95% level of confidence to ascertain statistical significance.

Chapter 3: Results and Discussion

Physical Effects of Matrix on Migration

Since HA is the most common glycosaminoglycan in the brain[5] and collagen can mimic blood vessels found in the brain, an HA/Collagen composite hydrogel was chosen to mimic the brain stroma. Thus, this composite hydrogel model exhibited fibrillar architectures resulting from fibrillar collagen formation and brain ECM resulting from the flat-sheet like HA.[26] Fiber density decreased with higher HA concentrations (Figure 3). This implies that HA interrupts collagen fiber formation. Composite HA/Collagen hydrogels provide the ability to tune HA or collagen concentrations, and hence mechanical properties. Thus, the composite hydrogel model is a step closer to ECM composition of the brain, which is complex and heterogeneous in nature.
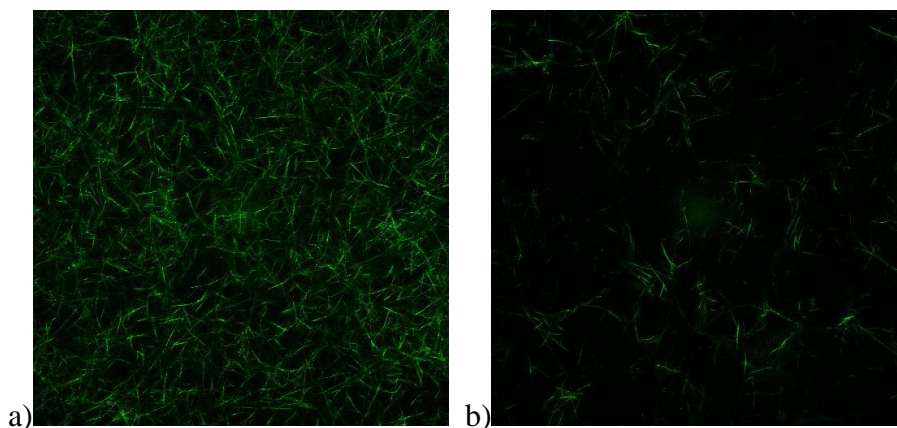

Figure 3: Confocal reflectance microscopy images of a a) 1% wt/vol HA/collagen hydrogel and a b) 2% wt/vol HA/collagen composite hydrogel.

From tests done previously by Rao *et. el*, it was found that a higher HA concentration increased the stiffness of the composite hydrogel matrix. It was found that the elastic modulus of the hydrogels increased significantly at HA concentrations of above 1%. (Figure 4) This indicates the hydrogels are becoming stiffer as the HA is added. With the ranges of elastic modulus of the composite hydrogel falling in the ranges of brain and mesenchymal tissue, this implies that the composite hydrogel is both chemically and mechanically tunable.
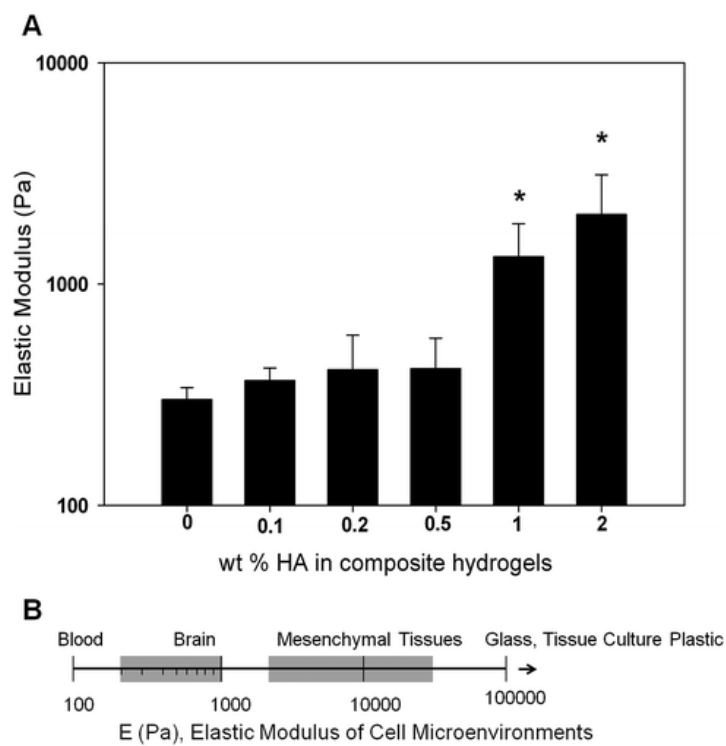

Figure 4: a) Elastic modulus of collagen and HA/Collagen composite hydrogels. b) Elastic modulus of cell microenvironments[26]

However, before moving forward in developing the proposed BBB model, we sought to evaluate the influence of these models on cell migration behaviors. These

models also create a closer physiologic representation of the brain microenvironment than a 2D model. Studies of the influence of tumor microenvironment, such as that found in the composite hydrogel matrices evaluated here, on the migration of cells are therefore needed prior to implementation in BBB models.

Thus, we investigated the impact of composite hydrogels of differing HA concentrations (0, 0.1, 0.2, 0.5, 1, 100 % wt/vol HA) on cancer cell migration velocity. Matrigel, a basement membrane matrix, [28] was also used as a control to compare the characteristics of MDA-MB231 breast cancers cells in breast mimetic tissue to that in brain mimetic tissue. Cell migration of MDA-MB231 cells was characterized by time-lapse fluorescent microscopy. There was a slight decrease in migration velocity as the HA concentration is at 0.5% (Figure 5), suggesting that HA may inhibit the migration in MDA-MB231 cells. Another possible explanation is that HA interferes collagen fiber formation and thus inhibits cell migration via the loss of cell adhesion footholds formed with the collagen fibers. However, since there is no consistent statistical significant trend along the titrated HA concentrations, more replicates would have to be done to increase statistical power.

An important part of the metastatic process involves the cells undergoing epithelial to mesenchymal transition (EMT).[1] EMT results in reduced cell-cell adhesion, which helps induce intravasation in which the cancer cells leave the primary site and enter into circulation in the blood stream. In mesenchymal migration modalities, the cell needs to adhere to the ECM and employs matrix metalloproteases to cleave this ECM, permitting migration to a nearby site.[29] Since breast cancer cells are likely to migrate via

the mesenchymal modality, the lack of cell adhesion to the matrix would then cripples the cell's ability to migrate. This could possibly explain the observed dip in migration velocity as HA concentration reached 0.5%. This observed decline as HA concentration increases could also be explained by the degree of crosslinking in the hydrogel matrix. As the concentration of HA increases, the degree of crosslinking increases too, and that has been shown to confer a higher resistance to invading MDA-MB231 cancer cells.[30] This could also be attributed to chemical factors where HA is inhibitory on cell migration. Thus, a high HA concentration might lead to a decline in cell migration velocities.

Moreover, it was observed that the migration velocities for breast cancer cells in Matrigel differed significantly from that of cells in HA/Collagen composite hydrogels, implying that the behavior of breast cancer cells in the brain can differ from that in its native breast environment. Studies such as these that elucidate such differences caused by the microenvironment might hold the key to preventing or treating metastases.
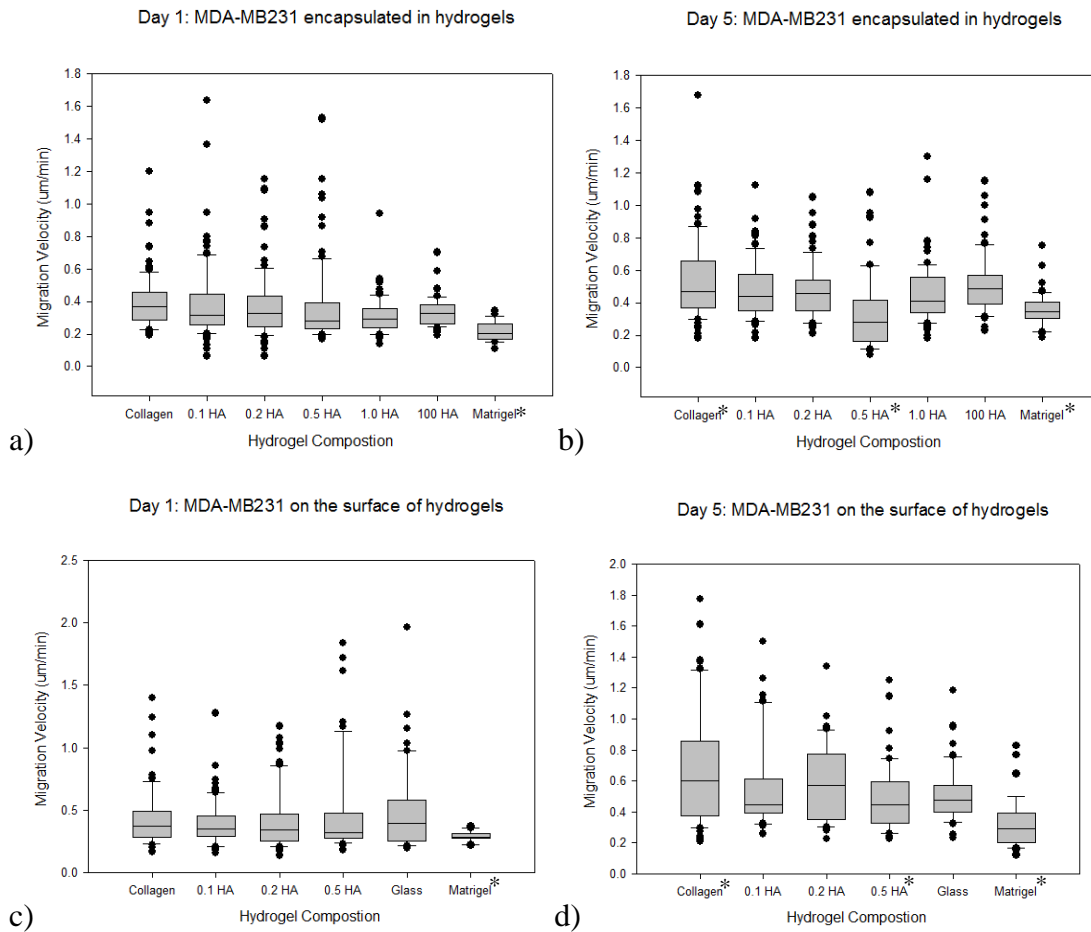
Figure 5: a) Migration velocity of MDA-MB231 when encapsulated in hydrogels at day 1. b) Migration velocity of MDA-MB231breast cancer cells when encapsulated in hydrogels at day 5. c) Migration velocity of MDA-MB231 when suspended on the surface of hydrogels at day 1. d) Migration velocity of MDA-MB231 cancer cells when suspended on the surface of hydrogels at day 5. Statistics are conducted at a p level of 0.05

From our observations (Appendix B), it appears that an HA concentration > 0.5% wt/vol inhibits MDA-MB231 proliferation. This is interesting as it has been found that invasion and proliferation are 2 mutually exclusive phases, i.e., cell cannot invade and proliferate at the same time. [31] Thus, our observation that MDA-MB231 cancer cells

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encapsulated in composite hydrogels at lower HA concentrations can proliferate and migrate effectively seems to contradict the "go or grow" model proposed previously. This could stem from the high HA concentrations leading to a higher matrix stiffness[26], which could then create a mechanical barrier for cancer cells to proliferate or invade. This is because HA has the ability to bind to water to create hydrous channels in the ECM,[32] which would aid migration, but as HA concentration increases the high HA sheet density and degree of crosslinks might reduce the amount of hydrous channels for migration. Moreover, it has also been shown that HA contributes to the stiffness of the hydrogel, as gels with high HA concentrations tend to be stiffer.[26] That, coupled with the migration of cancer cells being affected by the porosity of its 3D environment[32], would lead to the observed decrease of migration as HA concentration increased. On the other hand, this could also be due to the hydrogel creating a hostile environment that promotes cell apoptosis. Thus, an MTT cell proliferation assay should be conducted before concrete conclusions could be made.

Encapsulated vs. Surface Migration

Having shown that the physical properties of the matrix can impact the migration of cancer cells, we next sought to ascertain breast cancer cells response to the matrix in 2D vs. 3D conditions. Thus, we encapsulated cancer cells in composite hydrogels (3D) and compared these results to cell suspended on the surface of the hydrogels (2D). A schematic of this study can be found in Appendix A.
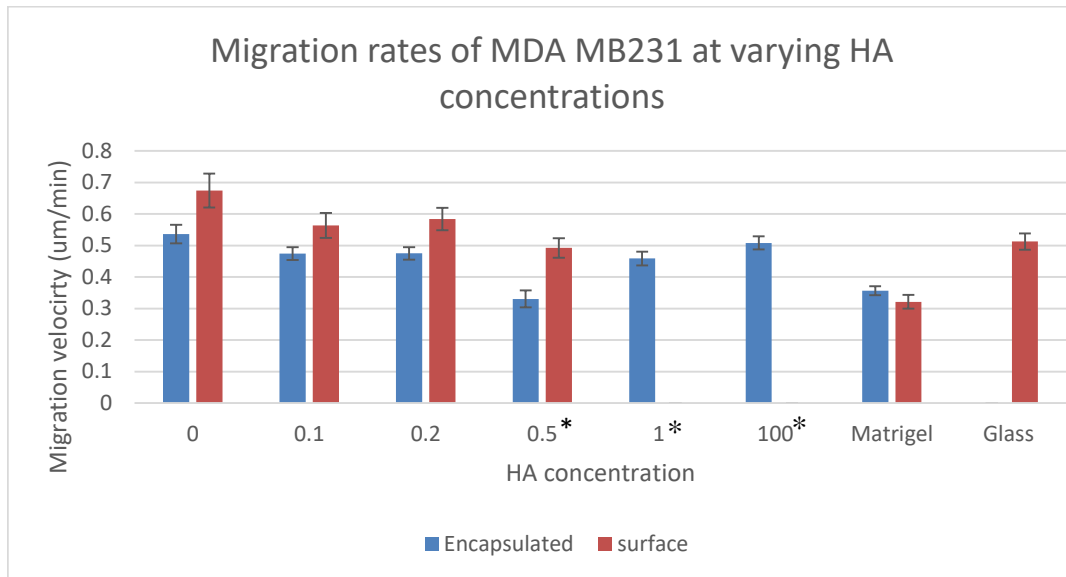
Figure 6: Migration rates of MDA-MB231 encapsulated and suspended on the surface (Day 5).

There was a significant difference at a 95% confidence interval between the encapsulated and the surface conditions on day 5 (Figure 6) that the cells are moving faster in the surface condition. This might be because of the cells in the surface condition not being influenced by the matrix stiffness or density. Without both the chemical and mechanical conditions of the hydrogel negatively impacting the migration, it is able to move faster than the encapsulated condition where both the chemical and mechanical effects of the gel is slowing down the migration. Surface tests were not performed at concentrations > 1% HA because, the MDA-MB231 cancer cells were unable to attach firmly to the hydrogel under these conditions. Cells at very high concentrations of 100wt% HA clumped together. Thus, this implies that the cells are only attaching to collagen fibers and needs it to migrate.

The difference in migration rates between the encapsulated and surface conditions at HA concentrations of above 0.5% wt/vol could also be explained by cell adhesion. Since cell adhesion was adversely impacted by the presence of HA, the increase in HA concentrations in the composite hydrogel constructs would lead to reduced cell-matrix adhesion. Thus, the observed spike in migration velocity in the surface condition might result from lower cell-matrix adhesion aiding migration in the surface condition or the cancer cells responding to the lack of cell adhesion footholds by moving to seek out better cell-matrix contact. Cell migration might be inhibited in the encapsulated condition as the matrix stiffness might obstruct migration. This shows the importance of comparing 3D and 2D behaviors in invasion studies. There was no significant difference between migration rates in day 1.

Moreover, it is interesting that this difference in migration rates is not seen in the Matrigel control. This suggests that a chemical pathway could be at work here, counteracting the physical effects of the increased ECM stiffness experienced by MDA-MB231 cancer cells while being encapsulated.

Day 1 vs. Day 5 Migration

There was also an observed difference between the Day 1 and Day 5 migration rates for encapsulated cells (Figure 7).
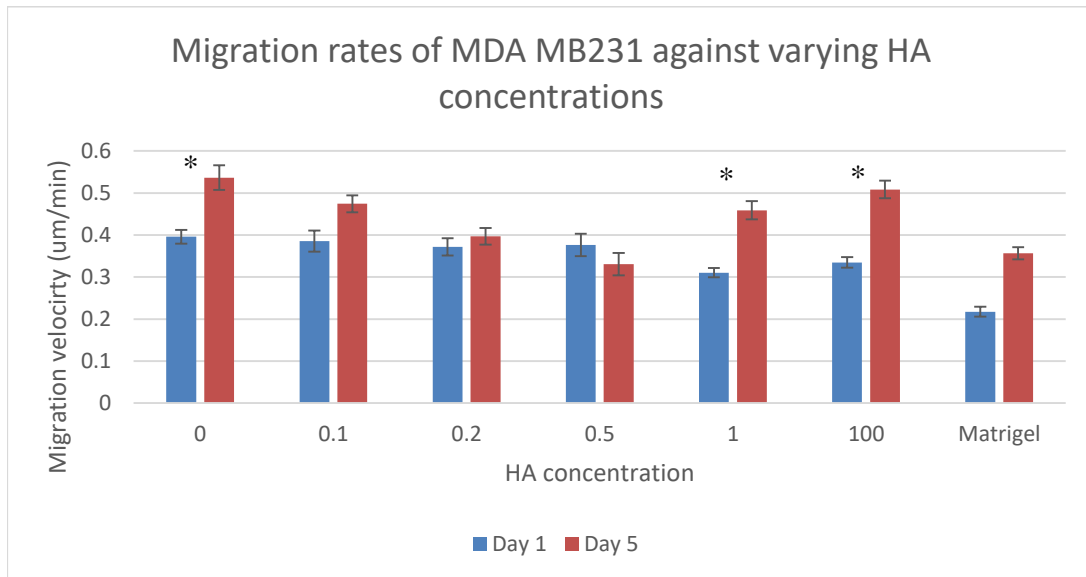
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Figure 7: Migration rates of encapsulated cells MDA-MB231 during Day 1 vs Day 5

There was a statistically significant difference in the migration rates between day 1 and day 5 for the collagen hydrogels and composite hydrogels > 1% HA wt/vol. This is consistent with a study that showed that cells remodel ECM and create paths that can be used for migration.[29] Hence, it is possible that the increased stiffness resulting from higher HA concentrations decreased over time as cells produced MMPs and remodeled the ECM. This has implications on the use of this hydrogel for scaffolding, as scaffold degradation is an important factor to consider when choosing the scaffolds for tissue engineering purposes or for building a better brain mimetic in the BBB model.

Chapter 4: Conclusions

A 3D hydrogel model was proposed to mimic conditions found in the BBB to improve study of breast-to-brain metastasis. This model offers several advantages to Transwell® inserts, the current gold standard, in that it has no rigid membrane barriers which may be more physiologically relevant.[4] HA and collagen are the two most common ECM components in the brain. Hence, they were chosen to mimic the brain stroma. It was hoped that these composite hydrogels would add another layer of complexity to the proposed BBB model and offer a more physiologic representation of the brain stroma. Thus, a cell invasion study in which MDA-MB231 cell migration under encapsulated and surfaced conditions was studied using HA/collagen hydrogels of varying HA composition. It was found that high levels of HA stunted cell proliferation, adhesion to the surface of the gel, and cell migration velocity. Moreover, migration velocities increased over time, possibly as the cancer cell remodeled its ECM or as the cells responded to the increased stiffness of the matrix by activating a Rho-mediated pathway[33], suggesting that hydrogel degradation is an important factor to consider going ahead.

The BBB is a protective layer between the circulatory system and the brain. It is comprised of endothelial cells, pericytes, and astrocytes and is characterized by its tight gap junctions. Thus, the next step of this project would be to investigate the integrity of our proposed BBB mimetic using both immunofluorescence and transendothelial

electrical resistance (TEER) techniques.[34] Should this model prove to be effective, it would be ideal for revealing the pathways governing breast-to-brain metastasis which may provide novel targets for therapeutics. These models could also be used to develop therapeutics, since the main challenge in dealing with brain metastases is the inability of drugs to penetrate the BBB. Moreover, this model could also be potentially expanded to investigate brain-tumor barriers (BTB) that form in brain metastases. BTBs are formed once cancers cells have successfully invaded the BBB. Little is known about these barriers, except that they have enhanced permeability and retention of fluids.[35]

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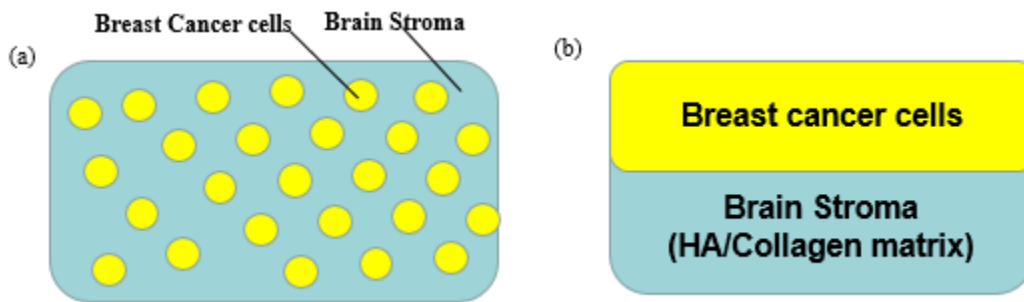Appendix A: Pictorial representation of experiments

Figure 8: Pictorial Representation of a) the encapsulated condition and b) surface condition used to investigate the physical effects of the HA/Collagen Matrix on migration.

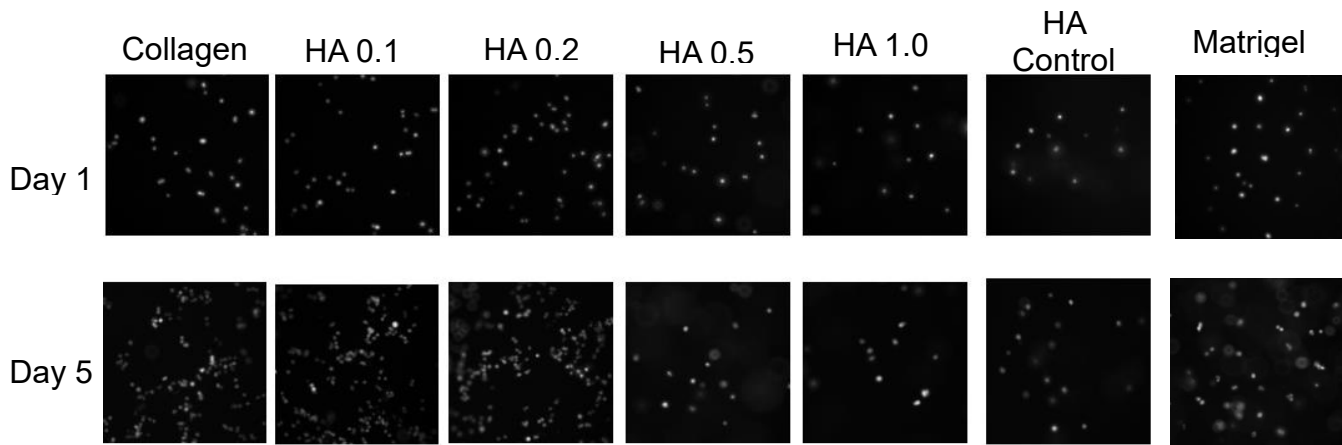Appendix B: Still images of MDA-MB231 in composite hydrogels

Figure 9 : Fluorescent images of MDA MB-231 cells in encapsulated in composite hydrogels at Day1 and Day 5
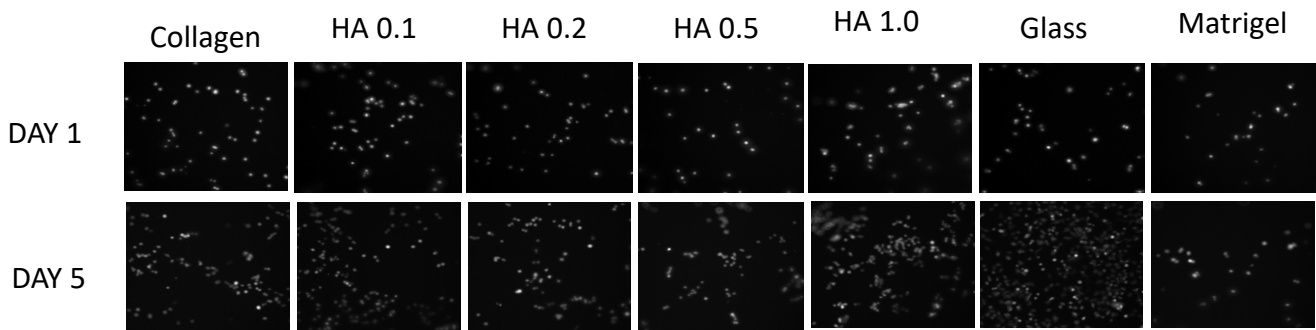

Figure 10: Fluorescent images of MDA MB-231 cells suspended on the surface of composite hydrogels at Day1 and Day 5