Effect of Tannic Acid Crosslinking on the Mechanical Properties of Collagen Scaffolds and Melanoma Cell Proliferation

by

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We, the undersigned committee, hereby approve the attached thesis, “Effect of Tannic Acid Crosslinking on the Mechanical Properties of Collagen Scaffolds and Melanoma Cell Proliferation,” by Christopher J. Bridgeman, be accepted as fulfilling in part the requirements for the degree of Master of Science in Biomedical Engineering

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Abstract

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Metastatic melanoma is responsible for nearly 10,000 deaths in the United States each year. While the prognosis for patients with localized early stage melanoma cancer is good, the chances of survival for patients with late stage metastatic melanoma are only 16%. Matrix stiffness has been shown to have a profound effect on tumor metastasis for breast cancer, but little is known about its role on melanoma metastasis and progression. Similarly, the therapeutic effect of tannic acid (TA) has been extensively characterized for soft breast and leukemia cancers, but not for melanoma. The goal of the current study was to investigate the synergistic effects of TA and collagen matrix stiffness on the proliferation of A375 melanoma cells. We hypothesized that TA will preferentially inhibit the proliferation of A375 melanoma cells compared to non-cancerous NIH 3T3 fibroblasts. Further, we hypothesized that TA will have a differential effect on soft
collagen gels vs. stiff collagen gels. Uncompacted (UC) collagen gels (soft) were prepared using a conventional method to induce collagen fibrillogenesis. An electro-compaction method was employed to densify collagen solutions and synthesize electrochemically compacted (ECC) collagen gels (stiff). Different concentrations of TA were used to crosslink UC and ECC collagen gels and assess its effect on gel morphology, mechanical properties and cellular response. SEM imaging showed that TA crosslinking resulted in the merging of collagen fibrils and decrease in pore size of both UC and ECC collagen gels. Tensile test results showed that TA crosslinking significantly (p < 0.05) improved the strength and modulus of ECC collagen gels. Results from Alamar blue assay showed that TA solubilized in the culture medium inhibited the proliferation of both A375 melanoma cells and NIH 3T3 fibroblasts. However, TA more significantly decreased (p < 0.05) the proliferation of A375 cells compared to NIH 3T3 fibroblasts on UC collagen gels. Further, A375 cell proliferation was more significantly reduced (p < 0.05) on TA crosslinked UC collagen gels compared to TA crosslinked ECC collagen gels. On the other hand, TA had a similar effect on both cell types when cultured on ECC collagen gels. In conclusion, results from this study suggest that TA incorporated into UC gels may preferentially affect melanoma cells, and that matrix stiffness is an important driver of tumor proliferation and progression.
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Dedicated to my parents and my brothers
Chapter 1

Introduction

1.1 Melanoma

Metastatic melanoma is considered the most aggressive form of skin cancer, and is responsible for nearly 10,000 deaths in the United States each year. While the prognosis for early stage localized melanoma cancer is good, the chances of survival for patients with late stage metastatic cancer is only 16%. Within the epidermis there are three main cell types: squamous cells, basal cells, and melanocytes. Melanocytes produce melanin pigment, whose function is to protect the hypodermis from Ultraviolet-B (UV-B) damage by scattering harmful UV rays. However in the process the melanocytes can become irreparably damaged leading to uncontrolled growth which is why melanoma often presents as a dark mole. The absorption of the UV light can have a damaging effect on the cell DNA which can induce melanoma via two main pathways. Oncogenes are regulatory genes in the cell responsible for cell growth and division. When oncogenes become damaged they have the potential to induce amplified cell growth which can lead to aggressive tumors. Tumor suppressor genes, antioncogenes, are responsible for inducing cell death to prevent the formation of a tumor, but they may be ineffective if they are overwhelmed from damaged oncogenes. The second, more common, pathway is one where the antioncogenes becomes damaged and fails to induce cell...
death at the appropriate time. These gene mutations are not mutually exclusive, and can occur in combination with other genetic changes. Melanoma cancer may form anywhere on the body that contains melanocytes, pigment producing cells, however generally cases are restricted to the epidermis and eyes.\textsuperscript{5,6}

The stages of melanoma cancer, its severity, can be characterized by tumor thickness, mitotic rate, and metastasis.\textsuperscript{7} Tumor thickness, known as the Breslow measurement, is a measurement of total thickness of the tumor, and subdivides skin cancer into five stages based on depth of the tumor.\textsuperscript{8,9} This is important in characterizing the potential for the tumor to reach a main blood source and metastasize (spread).\textsuperscript{10} Thicker tumors erode the epidermis and underlying skin layers which can lead to metastasis of the skin cancer to local and distal organs.\textsuperscript{11-13} Mitotic rate is the number of cancerous cells dividing in a tumor, and can be used to more reliably characterize the aggressiveness of the melanoma cancer.\textsuperscript{14} Tumors with a higher mitotic rate are more aggressive, later stage, and have a lower survival rate.\textsuperscript{14} Finally, ulceration is the breakdown of the epidermis over the melanoma, and is a general predictor for the severity of the cancer although it is considered a poor prognostic sign.\textsuperscript{14,15}

Although the prognosis for patients with early stage melanoma (I-III) is good, metastatic melanoma (IV-V) is much harder to overcome due to its aggressive nature. Metastasis is the uncontrolled growth and spreading of tumor cells to local or distal parts of the body, making it difficult to isolate and target.
Although chemotherapeutic agents have the ability to target a large portion of the body, melanoma cancer have developed a high level of resistance to these agents due to its ability to repair damage to its cellular pathways, and ability to clear toxins from the cells.\textsuperscript{16-18} Therefore it is necessary to develop more sophisticated models that not only investigate the direct effects of chemotherapeutic agents, but also explore the influence of surrounding tissues and ECM components on cancer metastasis and progression.

\subsection*{1.2 Dynamic Reciprocity and ECM influence}

The extracellular matrix (ECM) is a complex network of macromolecules whose chemical composition and mechanical properties have been shown to drive cell growth, proliferation, and apoptotic rate through the concept of dynamic reciprocity put forth by Bissell et al.\textsuperscript{19,20} Dynamic reciprocity is a term to describe the feedback interactions between the ECM and cells. Bissell et al. first demonstrated that mammary epithelial cells induced a circulating feedback loop where cells would increase the stiffness of the ECM to modulate gene expression via transmembrane proteins and cytoskeletal components involved in cell proliferation. Subsequent research was able to show that local cell signaling or ECM matrix composition could induce activation of oncogenes, or other genetic mutations, that would lead to tumors through the increase of matrix
metalloproteinase enzymes responsible for collagen degradation.\textsuperscript{21,22} Thus it was shown that ECM composition is controlled by localized cell secretion, and that the ECM exerts a mechanical and chemical influence on the morphology and gene expression of mammary cells which could potentially cause them to become carcinogenetic.\textsuperscript{23} Tumor remodeling of the ECM increased stiffness due to collagen crosslinking which promotes cell migration through the increase in number of focal adhesions not only for breast cancer but also for prostate, and hepatic cancer.\textsuperscript{23,24}

Traditional 2-D cell cultures on tissue culture-treated plastic (TCTP) do not accurately model the native ECM-tumor cell microenvironment, and it is not possible to decouple the variable effect of stiffness on cancer cell proliferation with TCTP.\textsuperscript{25} Collagen is a major component of the dermis ECM where metastatic melanoma frequently occurs, and hence is a biomaterial of immense interest for the development of tissue-engineered scaffolds as in vitro models for cancer research.\textsuperscript{26} Collagen hydrogels and scaffolds are versatile in the sense that their complexity can be easily modulated through the addition of other ECM components such as elastin, fibronectin, glycosaminoglycans (GAGs), and laminin to be able to better mimic the native ECM composition in the tumor microenvironment. Furthermore the ability to modulate the stiffness of collagen gels has improved in the last fifty years through the use of crosslinking agents and development of novel scaffold fabrication methodologies.\textsuperscript{27,28} Many studies have recently investigated the proliferation and morphology of soft sarcoma cancer cells on collagen-based
scaffolds. However despite crosslinking, traditional uncompacted scaffolds comprise of loosely packed collagen fibrils and hence are still relatively soft substrates, and therefore there is a need to improve the stiffness of collagen matrices through the development of alternative methods to form denser collagen gels.

1.3 Electrochemical Compaction Method

Electrochemical compaction of collagen fibrils is one such method used to modulate the stiffness of collagen gels. Based on the principles of isoelectric focusing, the electrochemical method was first developed in 2008 to synthesize highly aligned collagen bundles for tendon tissue engineering applications. Recent work has shown that this method can also be used to synthesize dense sheets of collagen with significantly higher stiffness compared to traditional collagen gels by subjecting the collagen solution to an electrocompaction process using planar electrodes. The resultant electrochemically compacted (ECC) collagen fibers and gels have been utilized in a wide variety of tissue engineering applications for the repair and regeneration of diseased or damaged tendon, cornea, and bone. However, ECC gels have yet to be utilized as models to understand tumor progression. The ability to modulate the stiffness of collagen hydrogels using the electrocompaction process will allow for a thorough investigation of the effect of
matrix stiffness on A375 cell proliferation. Improving the stiffness and complexity of collagen gels is critical to create more realistic models of the ECM matrix that will better allow scientists to model and predict tumor behavior and response to independently controlled variables.

1.4 Tannic Acid

Polyphenols are an important class of molecules that have antioxidant properties, are anticarcinogenic, and whose effects have been shown to be potentially selective to cancer cells. Polyphenols have been shown to interact with cell functions by disrupting signaling cascades, interfering with membrane and intracellular receptors, and interacting with enzymes involved in tumor metastasis. Tannic acid (TA) is a hydrolysable tannin that is known to crosslink collagen fibrils through hydrogen bonding and hydrophobic effects. TA has been used previously as a crosslinking agent for aortic valve replacements, and has been extensively studied due its practical application in wound healing. TA has recently been shown to preferentially inhibit MCF-7 breast cancer cell proliferation compared to non-cancerous differentiated D1 cells. A follow up study supported this conclusion, and showed that TA induced a higher apoptotic rate in MCF-7 and MDA-MB-231 breast cancer cell lines compared to non-cancerous MCF10A breast cells through the activation of caspase cascades. These results were reinforced in a
study by Chen et al. which showed that TA increased the apoptotic rate of acute myeloid leukemia (AML) HL-60 cells through the activation of caspase cascades.\textsuperscript{36} Furthermore, Natarajan et al. showed that TA crosslinked collagen scaffolds did not significantly affect NIH 3T3 fibroblast proliferation, indicating that TA may be used to target the disruption of cancerous cell proliferation while having a minimal effect on healthy normal human body cells.\textsuperscript{39} However the results of these studies have not been extensively extrapolated past breast and leukemia cancers, so there is a need to investigate the effects of TA on other cancer types like melanoma.

1.5 Problem Statement

Although the characterization of melanoma growth is well defined, and the general UV induced mutagenic pathway has been identified, there is a poor understanding of the physiochemical drivers that affect tumor growth in the human body. Matrix stiffness has been shown to have a significant effect on tumor metastasis for breast cancer, but little is known about its role on melanoma metastasis and progression. Further, TA has been shown to be an effective anticancer agent in breast cancer studies but has not yet been explored for melanoma. Therefore, there is a need for in vitro models to synergistically characterize the effects of matrix stiffness and TA on melanoma metastasis. The main goal of the current study was to investigate the anticarcinogenic properties of
TA on melanoma by exposing A375 melanoma cancer cells to different doses of TA delivered via soft and stiff collagen gels, and assess its effect on A375 melanoma cell morphology and proliferation. TA was delivered in three different ways: 1) solubilized in the culture medium, 2) matrix bound on uncompacted (UC) collagen gel (soft), and 3) matrix bound on electrochemically compacted (ECC) collagen gels (stiff). NIH 3T3 noncancerous cell line was used as control in all the experiments. Overall, the outcomes of this study will demonstrate the therapeutic effects of TA on A375 melanoma cell proliferation, with a secondary focus on assessing A375 response to varying gel stiffness.

1.6 Hypotheses

There are four main hypotheses in this study:

**H1:** TA crosslinking will induce morphological changes to UC and ECC collagen gels.

**H2:** TA will increase strength and stiffness of ECC collagen gels.

**H3:** Exposure to TA will induce changes in cell morphology and more significantly reduce proliferation of A375 melanoma cancer cells compared to non-cancerous NIH 3T3 fibroblasts.

**H4:** TA will induce a differential effect on A375 melanoma cancer cell proliferation on UC (soft) vs. ECC (stiff) collagen matrices
The effect of TA crosslinking on the morphology of UC and ECC collagen gels was assessed using scanning electron microscopy. Monotonic tensile tests were performed to evaluate the effect of TA crosslinking on the mechanical properties of ECC collagen gels. Cell morphology on uncrosslinked and TA crosslinked UC and ECC collagen gels was assessed by staining the cell cytoskeleton and imaging using confocal microscopy. Finally, Alamar blue assay was performed to assess the effects of TA and matrix stiffness on cell proliferation.

### 1.7 Goals

To test the hypotheses of the study, the specific aims of this project were:

1. Assess the effects of TA crosslinking on UC and ECC collagen gel morphology.
2. Quantify the amount of TA incorporated within UC and ECC collagen gels post crosslinking.
3. Investigate the effect of TA crosslinking on the mechanical properties of ECC collagen gels.
4. Assess the effects of soluble TA on proliferation of A375 cells and NIH 3T3 fibroblasts.
5. Assess the synergistic effects of TA crosslinking and matrix stiffness on morphology and proliferation of A375 melanoma cells and NIH 3T3 fibroblasts.
Chapter 2

Experimental Methodology

TA cross-linked UC and ECC collagen gels were fabricated to assess the synergistic effects of TA and collagen matrix stiffness on the morphology and proliferation of A375 metastatic melanoma cells and NIH 3T3 fibroblasts. This chapter describes the synthesis of UC and ECC collagen gels, the methodology employed for TA cross-linking of these gels, gel characterization methods, and methods for evaluating cellular response to soluble TA and TA crosslinked UC and ECC collagen gels.

2.1 Synthesis of Uncompacted Collagen Gels:

Acid soluble type-I collagen solution (Advanced BioMatrix; PureCol; San Diego, CA) was mixed with 10x PBS and 0.1 M sodium hydroxide in a volume ratio of 8:1:1. First, the collagen solution was mixed with 10x PBS and then the pH was neutralized (pH: 7.2-7.4) by the addition of 0.1 M sodium hydroxide. Fifty µl of the pH neutralized collagen solution was added to each well of a 96 well plate, and incubated at 37 °C for 1 hour to allow for the collagen molecules to undergo fibrillogenesis and form uncompacted (UC) collagen gels. The formation of UC gels was confirmed by visualizing the change in the solution from clear to opaque.
2.2 Synthesis of Electrochemically Compacted Collagen Gels:

ECC collagen gels were synthesized as previously described.\textsuperscript{33} Briefly, acid soluble type-I collagen solution was dialyzed against RO water for 24 hours with water changes every two hours for the first six hours. Dialyzed collagen solution was loaded into rubber molds, placed between two graphite electrodes, and an electric field (3V) was applied for 45 minutes. In the presence of an electric field, a pH gradient develops between the electrodes. Due to its amphoteric nature, the collagen molecules assume a positive charge close to the anode (acidic pH) and negative charge close to the cathode (alkaline pH). Since the electrodes have a like charge, the collagen molecules repel away from the electrodes to self-assemble along the isoelectric point where the net charge was zero. The resultant ECC collagen gel was collected from the cathode at the end of the process. Following this, the ECC collagen gels were incubated in 1x PBS at 37 °C for four hours to induce fibril formation.\textsuperscript{41} Finally, the ECC collagen gels were then washed for five minutes in DI water to wash off the PBS solution. Both UC and ECC collagen gels were prepared fresh for each experiment.
2.3 TA Crosslinking of UC and ECC Gels:

UC and ECC collagen gels were crosslinked with TA by adopting a protocol from previously published literature. Gels were crosslinked in freshly prepared TA solution for four hours at room temperature. Three different concentrations of TA (0.1 wt%, 1 wt%, and 10 wt%) were used. Gels incubated in water with no TA were used as controls. After crosslinking, the gels were washed four times in DI water for a duration of 15 min for each wash; this ensured all excess TA was removed. The resultant uncrosslinked and crosslinked UC and ECC collagen gels were used for all the experiments performed in this study.

2.4 Scanning Electron Microscopy of UC and ECC Gels

SEM analysis was performed to assess the effect of TA crosslinking on the morphology of the UC and ECC collagen gels. Gels were first dehydrated in ethanol-water mixtures (20%, 50%, 75%, 90%, 100%) for five minutes in each solution. Following this, the gels were soaked in a 1:1 (v/v) mixture of amyl acetate and ethanol for five minutes, and then immersed in 100% amyl acetate solution overnight. Finally, the gels were subjected to critical point drying (Denton DCP-1 Critical Point Dryer) by placing them in a mesh basket and exposing them to CO₂ flow for 45 minutes at a pressure of 950 psi. The container with the gels was then
exposed to warm water at 55 °C. This increased the pressure of the CO$_2$ to 1650 psi. As the pressure passed through 1050 psi the critical point was achieved. Gels were then removed under dry conditions to ensure that no moisture contaminated the samples. Gels were sputter coated with gold and imaged under the SEM.

### 2.5 Quantification of TA Incorporated into UC and ECC Gels

UC and ECC collagen gels (N=6/group/treatment) were prepared and crosslinked with TA as previously described. To quantify the amount of TA incorporated within the gels, gels were individually immersed in one milliliter (ml) of 1.0 molar (M) hydrochloric-acid (HCL) solution and incubated at 66° C for 24 hours to allow for complete dissolution. Hundred µl of solution from each sample was transferred into a 96 well plate in triplicate. The amount of TA present in the solution was determined by reading the absorbance at 273 nm (Spectramax, Molecular Devices) and comparing the readings with a standard curve generated using known concentrations of TA.

### 2.6 Mechanical Testing of ECC Gels

Monotonic tensile testing was performed to assess the effect of TA crosslinking on the tensile properties of ECC collagen gels (N = 12/group). UC collagen gels were very weak and could not be physically handled; therefore, the tensile properties of UC collagen gels were not investigated in this study. First, the
thickness of uncrosslinked and TA crosslinked (0.1%, 1% and 10%) ECC collagen gel (20 mm length x 5 mm width) was measured using a custom set up by placing the gel on a graphite electrode and adjusting a caliper until it contacted the surface of the scaffold. Contact with the scaffold was confirmed by monitoring the change in the resistance using a multimeter. Thickness measurements were performed at three distinct points along the length of the gel and the average value was used to calculate the crosssectional area (width x average thickness) of each sample. Following this, the ECC collagen gels were mounted onto plastic frames, and the ends of the gels were glued using Loctite® adhesive. The adhesive was allowed to dry for 45 minutes, and then the gels were rehydrated in DI water for 45 minutes prior to testing. Tensile tests were performed under load control (0.1 N/min) until failure (Q800 DMA, TA Instruments), and load and displacement data were recorded. Stress was calculated by normalizing the load data with the cross-sectional area. Strain was calculated by taking the ratio of the change in length to the original length of the sample. Stress-strain curves were generated and the ultimate tensile stress (UTS) and ultimate strain (US) was determined. Finally, tensile modulus was calculated by taking the slope of the steepest region of the stress-strain curve.
2.7 Cell Culture:

A375 melanoma cells and NIH 3T3 fibroblasts (noncancerous control) were used in this study (ATCC). Cells were seeded at a density of 10,000 cells/cm² and cultured in high glucose Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin for 5 days for all the experiments performed in this study. At periodic intervals, cells were either fixed and stained for cell morphology assessment or subjected to Alamar blue (AB) assay to quantify cell proliferation.

2.7.1 Solubilized TA in Culture Medium: A375 melanoma cells and NIH 3T3 fibroblasts were exposed to varying concentrations of soluble TA (0, 50, 25, 5 g/L) over a 5 day culture period. At first, cells were seeded onto 96 well culture plates in regular growth medium (without TA), and allowed to attach for 24 hours. At day 1, the culture medium was replaced with culture medium containing TA (TA-DMEM). AB assay was performed (N=6/group/cell type) just before the introduction of TA-DMEM (day 1) and then at day 3 and day 5 to evaluate the effect of soluble TA on cell proliferation.

2.7.2 TA Crosslinked UC and ECC Collagen Gels: UC collagen gels were prepared in a 96 well plate, crosslinked with sterile solutions of TA (0.1%, 1%), and washed with copious amounts of water prior to seeding cells. ECC gels were prepared and crosslinked with TA (0.1%, 1%) as described earlier. Next, the ECC collagen gels
were sterilized by briefly immersing them in 70% ethanol solution followed by extensive rinsing in sterile 1x PBS. Sterile ECC collagen gels were placed individually in a 96 well plate. Uncrosslinked UC and ECC collagen gels were used as controls. Cells were seeded on to uncrosslinked and TA crosslinked UC and ECC collagen gels and cultured for 5 days. At day 1, day 3, and day 5, AB assay was performed to assess the effect of TA crosslinking and matrix stiffness on cell proliferation (N=6/group/cell type). Gels without cells (n=3/group) were used as blank for the AB assay. Additionally, at day 1 and day 5, UC and ECC collagen gels were fixed, and the cells were stained for cell morphology assessment (N=3/group/cell type).

2.8 Evaluation of Cell Proliferation

AB assay (Life Technologies #1025) was performed to: a) evaluate the effect of soluble TA on cell proliferation, and b) evaluate the synergistic effects of TA and matrix stiffness on cell proliferation. AB solution was warmed to room temperature slowly, and then mixed with DMEM (1:9 AB-DMEM). All AB work was done under low light conditions to prevent light contamination of the assay. Briefly, preexisting DMEM was removed from each of the wells and 120 microliters of AB-DMEM solution was added to each well. The AB-DMEM solution was incubated for 2 hours at 37 °C, and then 100 microliters of AB-DMEM solution from each well was pipetted into a standard 96 well plate for
analysis with a spectrophotometer. Plates were covered with tinfoil to prevent light contamination of the samples. The remaining 20 microliters of AB-DMEM solution was removed from each well, and replaced with fresh DMEM. Fluorescence measurements were recorded by using a M2e Spectramax plate reader (Molecular Devices) with an excitation wavelength of 555 nm and emission wavelength of 595 nm. Cell number was quantified by comparing the fluorescence measurements with a standard curve generated using known number of cells. Fold increase was calculated by normalizing the cell number at each time point with the cell number at day 1.

2.9 Evaluation of Cell Morphology

To assess the effect of TA crosslinking on cell morphology, cells seeded on UC and ECC collagen gels were washed twice with PBS for five minutes and fixed in a solution of formaldehyde (3.7%), PBS (1x), and Triton X-100 (0.05%) for 15 minutes at room temperature. Cells were then washed with PBS (1x) solution 2 times for 2 minutes each wash, and incubated in permeabilization buffer (0.2% Triton X-100 in PBS) for 15 minutes. Following this, the gels were washed with PBS and incubated in blocking buffer (PBS containing 0.05% Triton X-100) for 30 minutes at room temperature. The cell cytoskeleton was then stained with a working solution of AlexaFluor 488-Phalloidin (1:25 in 1x PBS) at room temperature for 30 minutes, washed and imaged using a confocal microscope.
(Nikon). Oil immersion was used to obtain high magnification (20x) confocal images to assess the morphology of the cells on uncrosslinked and TA crosslinked UC and ECC collagen gels.

2.10 Statistics

All statistics were performed using the program R. A Shapiro-Wilk test (p>0.05) was used to test for normality and a Bartlett test (p>0.05) was used to test for homogeneity of variance. Mechanical testing data was analyzed using a one way anova (p<0.05). For the cell proliferation experiments, data from multiple experiments were combined by normalizing the data from each experimental run to the day 5 0 mg/ml TA or day 5 uncrosslinked collagen gels. The individual normalized data points from all experiments were then multiplied by the mean value of the day 5 0 mg/ml TA or uncrosslinked collagen gels across experiments to obtain a combined data set of n ≥ 12 per group. A one way ANOVA was used to analyze data for the TA quantification experiment and cell proliferation studies. A Tukey-HSD test was used to test for significance between groups. Statistical significance was set at p < 0.05.
Chapter 3

Results

3.1 Assessment of Scanning Electron Microscopy of UC and ECC Gels

SEM analyses revealed that TA crosslinking resulted in significant changes in the morphology of both UC and ECC collagen gels (Fig. 3.1). Specifically, TA crosslinking induced merging of collagen fibrils in both UC and ECC collagen gels resulting in increased fibril thickness and decreased pore size in crosslinked gels compared to uncrosslinked gels. Further, while the morphology of ECC collagen gels crosslinked with 0.1 mg/ml and 1 mg/ml TA was comparable (Fig. 3.1F and 3.1G), the collagen fibrils appeared to be completely merged with a scaly appearance when crosslinked in 10 mg/ml TA (Fig. 3.1H). Similar morphological changes were also found in crosslinked UC collagen gels with a greater extent of fibril merging observed in the 10 mg/ml TA (Fig. 3.1B-D). When comparing the UC and ECC collagen gels, the ECC collagen gels exhibited thinner collagen fibrils and smaller pore size compared to UC collagen gels as a result of densification due to the electrochemical compaction process.
3.2 Quantification of TA Incorporated into UC and ECC Gels

The average amount of TA incorporated into crosslinked UC collagen gels and ECC collagen gels in 0.1 mg/ml solution was comparable (Fig. 3.2). Specifically, the amount of TA incorporated into UC collagen gels and ECC collagen gels in 0.1 mg/ml crosslinking solution was 0.021 mg and 0.027 mg, respectively. Similarly, the average amount of TA incorporated into UC collagen gels and ECC collagen gels in 1 mg/ml crosslinking solution was also comparable with 0.062 mg TA and 0.068 mg TA, respectively (Fig. 3.2). Together, these results indicate that changing the initial concentration of TA resulted in a significant difference (p<0.05) in the amount of TA incorporated into UC and ECC collagen gels. However, when comparing the two gel types for each TA concentration, no
significant differences were observed confirming that the amount of TA incorporated in both gels was the same.

![Graph showing quantitative TA concentration](image)

**Figure 3.2:** Quantification of amount of TA incorporated into UC and ECC collagen gels. Results indicate that the amount of TA incorporated into UC and ECC collagen gels for each TA concentration was comparable. Significance between crosslinking solution denoted by bar. Note that there was no significant difference in incorporated TA per TA Concentration treatment.

### 3.3 Assessment of Mechanical Properties of TA Crosslinked ECC Gels

Results from monotonic tensile testing revealed that TA crosslinking significantly altered the mechanical properties of ECC collagen gels (Fig. 3.3). Typical stress-strain curves for different concentrations of TA are shown in Fig. 3.3A. TA crosslinking significantly increased the ultimate tensile stress (UTS) of ECC collagen gels (Fig. 3.3B). Specifically, crosslinking with 1 mg/ml and 10 mg/ml TA resulted in a significant increase in UTS of ECC collagen gels (p < 0.05)
compared to 0.1 mg/ml TA and uncrosslinked (0 mg/ml TA) groups. Further, UTS of ECC collagen gels after crosslinking in 10 mg/ml TA was significantly higher (p < 0.05) than 1 mg/ml TA. There was no significant difference in the UTS of ECC collagen gels between 0 mg/ml and 0.1 mg/ml TA, or between 0.1 mg/ml and 1 mg/ml TA. Results for ultimate strain (US) followed an opposite trend to that observed for UTS such that the US of crosslinked ECC collagen gels was significantly lower (p < 0.05) than uncrosslinked ECC collagen gels (Fig. 3.3C). Tensile modulus (TM) of ECC collagen gels crosslinked with 1 mg/ml TA was significantly higher (p < 0.05) that 0 mg/ml TA (Fig. 3.3D). No significant differences in TM was observed between the remaining groups. Together, these results indicate that TA crosslinking increases the UTS and TM but decreases the US of ECC collagen gels.
3.4 Assessment of Solubilized TA on Cell Fold Increase

Results from AB assay showed that exposing the cells to soluble TA significantly decreased proliferation of both A375 cells and NIH 3T3 fibroblasts (Fig. 3.4 A-B). Cell proliferation in the presence of 5 g/L TA was comparable to that of no TA for both cell types indicating that exposing the cells to a low concentration of soluble TA had no effect on cell growth. However, increasing the soluble TA concentration to 25 g/L and 50 g/L resulted in a significant decrease ($p < 0.05$) in the proliferation of both cell types when compared to 0 g/L and 5 g/L. Further, a significant decrease ($p < 0.05$) in cell proliferation was also observed when comparing the 25 g/L TA and 50 g/L TA groups for both cell types. When
comparing the two cell types, the decrease in cell proliferation upon exposure to soluble TA was found to be comparable suggesting that there was no preferential effect of soluble TA on cancerous cells (Fig. 3.4C). Together, these results indicate that exposure to higher concentrations of soluble TA inhibited proliferation of both cell types.

**Figure 3.4:** Assessment of different concentrations of soluble TA on the cell proliferation using Alamar blue assay. A) A375 cells, and B) NIH 3T3 cells treated with soluble TA in DMEM. C) Day 5 cell proliferation ratio. Significant differences in cell number between groups at day 5 are indicated by horizontal lines (p < 0.05).

### 3.5 Assessment of Cell Proliferation on UC and ECC Gels

AB assay was also used to assess the synergistic effects of matrix stiffness and TA crosslinking on the proliferation of A375 cells and NIH 3T3 fibroblasts. Firstly, cells proliferated well on uncrosslinked UC collagen gels and ECC collagen gels (without TA) with significant differences in cell numbers (p < 0.05) on day 3 and day 5 compared to day 1 for both cell types. Upon crosslinking with 0.1 mg/ml
TA, significant differences in cell numbers (p< 0.05) was only observed between day 5 and day 1 for both cell types on UC and ECC collagen gels. Further, when the collagen gels were crosslinked with 1 mg/ml TA, no change in cell number over time was observed for both cell types on UC collagen gels indicating that both cell types ceased to proliferate on soft gels at high TA concentration (Fig. 3.5A, 3.5B). On the other hand, while no change in NIH 3T3 cell number was observed on ECC collagen gels crosslinked with 1 mg/ml TA (Fig. 5D), a significant decrease in A375 cell number (p < 0.05) was observed when comparing the cell numbers on day 3 and day 5 with day 1 (Fig. 3.5C).

Secondly, when comparing between UC (soft) and ECC (stiff) collagen gels, greater cell proliferation was observed (p<0.05) on uncrosslinked ECC collagen gels compared to uncrosslinked UC collagen gels for both cell types suggesting that cells proliferate faster on stiffer substrates (Fig. 3.5). Upon crosslinking with TA, a significant decrease in cell number (p < 0.05) was observed for both cell types on UC and ECC collagen gels (Fig. 3.5). However, the extent of reduction in A375 cell proliferation was greater on UC collagen gels compared to ECC collagen gels as indicated by a steeper slope of the black line compared to the green line in Fig. 3.6 indicating that TA more efficiently inhibits A375 cell proliferation on softer gels compared to stiffer gels. Thirdly, when comparing between cell types, TA crosslinking had a greater effect on A375 cells compared to NIH 3T3 fibroblasts on UC collagen gels as indicated by a steeper slope of the
black line compared to the red line in Fig. 3.6. On the other hand, on ECC collagen
gels, the effect of TA crosslinking was comparable on both cell types as indicated
by the similar slopes of the blue line and green line in Fig. 3.6. Overall, these
results indicate that TA has a more pronounced effect on A375 cell proliferation on
soft substrates compared to stiff substrates. Further, TA preferentially inhibits
A375 melanoma cancer cell proliferation compared to noncancerous NIH 3T3
fibroblasts on softer substrates; conversely, on stiffer substrates, TA has a similar
effect on both cell types.
Figure 3.5: Assessment of matrix-bound TA on cell proliferation on UC collagen gels (soft) and ECC collagen gels (stiff). A) A375 cells on UC collagen gels, B) NIH 3T3 cells on UC collagen gels, C) A375 cells on ECC collagen gels, D) NIH3T3 cells on ECC collagen gels. Significant differences in cell proliferation (p < 0.05) on day 3 and day 5 compared to day 1 within a specific TA concentration are denoted by an asterisk. Significant differences in cell proliferation (p < 0.05) between TA concentrations at day 5 are denoted by horizontal lines.
Figure 3.6: Day 5 cell number ratio for A375 melanoma cells and NIH 3T3 fibroblasts seeded onto UC and ECC collagen gels. Cell ratio was calculated by normalizing the day 5 cell number for all groups with the day 5 cell number on the uncrosslinked gel for each cell type on UC and ECC collagen gel separately.

3.6 Assessment of Cell Morphology on UC and ECC Gels

A375 cells seeded on un-crosslinked and 0.1 mg/ml ECC (stiff) collagen gels at day 1 had a more round, epithelial, morphology and were more spread compared to A375 cells seeded onto uncrosslinked and 0.1 mg/ml soft UC collagen gels (Fig. 3.7). A375 cells seeded on UC and ECC collagen gels crosslinked with 1 mg/ml TA were visibly smaller in size and were fewer in number compared to uncrosslinked and 0.1 mg/ml TA crosslinked gels (Fig. 3.7). On day 5, significantly higher number of cells was observed on uncrosslinked UC and ECC collagen gels indicating that cells proliferate well when no TA is present (Fig. 3.9). However, cell number was significantly lower on 0.1 mg/ml TA compared to uncrosslinked
UC and ECC gels corroborating the results from the AB assay. Further, cells on 0.1 mg/ml TA gels exhibited a shrunken morphology compared to day 1. Least number of cells were observed on 1 mg/ml TA gels. Further, these cells lacked spreading for both gel types which may be indicative that the cells were not viable.

NIH 3T3 fibroblasts exhibited spindle shaped morphology on uncrosslinked and 0.1 mg/ml TA crosslinked UC and ECC collagen gels at day 1 (Fig 3.8). However 3T3 cells had greater spreading on uncrosslinked and 0.1 mg/ml TA ECC stiff gels further indicating that the cells responded to changes in gel stiffness. Cell numbers appeared to increase for both UC and ECC uncrosslinked gel types from day 1 to day 5 (Fig. 3.10). Cells cultured on UC and ECC collagen gels crosslinked with 1 mg/ml TA were few in number and morphologically unhealthy looking on both day 1 and day 5, indicating that the higher dose of TA completely inhibits cell growth on UC and ECC collagen gels.

Together, these results indicate that greater cell spreading of both A375 melanoma cells and NIH 3T3 fibroblasts was observed on ECC collagen gels compared to UC collagen gels suggesting that matrix stiffness induces morphological changes in both cell types.
Figure 3.7: Day 1 A375 cell morphology on UC and ECC gels. Scale bar =100µm. Note the increased cell spreading on ECC gels.

Figure 3.8: Day 1 NIH 3T3 Morphology on UC and ECC gels. Scale bar =100µm. Note the increased cell spreading on ECC gels.
Figure 3.9: Day 5 A375 morphology on UC and ECC gels. Scale bar =100µm. Note the increase in cell proliferation on uncrosslinked (0 mg/ml) gels.

Figure 3.10: NIH 3T3 ECC Morphology. Scale bar =100µm. Note the increase in cell proliferation on uncrosslinked (0 mg/ml) gels.
Chapter 4
Discussion

This study is the first to attempt to characterize the synergistic effects of TA and collagen gel stiffness on melanoma cell (A375) proliferation. A previous study by Cordoves et al. investigated the effects of phenolics extracted from red wine and the sorghum flower on melanoma proliferation, however they did not specifically evaluate the use of TA. Furthermore this study not only explores the synergistic effects of TA and matrix stiffness but also seeks to decouple their two independent effects through the use of NIH 3T3 fibroblast cells as a control. Modeling the response of melanoma cells to variable gel compositions and stiffness is critical for developing effective treatments for melanoma cancer. This study lays the groundwork for predicting melanoma cell behavior as a factor of stiffness, while also exploring the potentially selective therapeutic treatment of TA for cancer cells over normal non-cancerous human cells.

Natarajan et al. found that collagen fibrils merged after collagen gels underwent TA crosslinking. They also concluded that merged collagen fibers took on a scaly appearance, a finding that is well supported by another study performed by Cass et al. which also conducted SEM on crosslinked collagen substrates. The crosslinked collagen substrates (10 mg/ml) imaged by Cass et al. (x4000) were similar in appearance to the 10 mg/ml UC gels imaged in this study (x27,000)
although Cass et al. was unable to capture the individual merging of fibrils. The SEM photos in this study were able to confirm that fibrils took on a scaly appearance, and capture individual fibril merging for both UC and ECC gels (Fig. 3.1). Natarajan et al. reported that TA increased pore size in collagen gels due to the merging of the mesh like fibril network, although the images in this study indicated an opposite trend where TA crosslinking reduced pore size. This indicates that although TA will induce fibril merging in collagen gels, pore size at the cellular level is highly dependent on how the collagen gels are prepared. This conclusion is further support by the images of TA crosslinked ECC gels, which had a smaller pore size compared to UC gels, and had a higher amount of fibril merging most likely due to the already compacted nature of ECC gels even before TA crosslinking (Fig 3.1 F-H). Crosslinked ECC collagen gels had a flatter surface topography compared to UC collagen gels which can also be attributed to the ECC process, further supporting the conclusion that collagen porosity and topography are a synergistic result of the concentration of TA crosslinking solution and gel formation process.

TA is an established crosslinking agent that utilizes hydrogen bonding and hydrophobic effects to crosslink collagen fibrils. Previously TA was used as a topical treatment for skin burns due to its resistance to bacteria and ability to stabilize damaged tissue. The results from this study show that TA improved the strength and stiffness of ECC collagen gels through the merging of collagen fibrils (Fig. 2). Heijmen et al., Koide et al., and Bedran-Russo et al. all found that TA crosslinking increased the modulus of their
collagen matrices, although their collagen matrices were inherently much stiffer and are not directly comparable to the gels analyzed in this study. Natarajan et al. found that TA crosslinking did not increase the tensile strength of their scaffolds, however their scaffolds were created using a collagen-TA foam mixture which created a microstructure substantially different from the UC and ECC collagen gels employed in this study. Natarajan et al. found that TA crosslinking did not increase the tensile strength of their scaffolds, however their scaffolds were created using a collagen-TA foam mixture which created a microstructure substantially different from the UC and ECC collagen gels employed in this study.

Cass et al. found that soluble TA equally affected MCF-7 and undifferentiated D1 cells seeded on TCTP. Results using soluble TA from this study are in agreement with the findings of Cass et al and revealed there was no difference in cell proliferation between A375 and NIH 3T3 cells in this study (Fig. 3.4), indicating that TA when delivered in a soluble form inhibits proliferation of both cancerous and non-cancerous cells. The lack of difference between lower concentrations of TA (0, 5 g/L) may indicate that a threshold amount of TA is required to induce a significant change in cell proliferation. Further investigation using intermediate concentrations of TA (e.g., 10 g/L) may reveal if TA in soluble form triggers a differential response in the proliferation of the two cell types. However, there are other important factors that need to be considered when utilizing TCTP as a culture matrix. Most importantly, TCTP is a 2D substrate and does not recapitulate the native 3D ECM environment found in the human body. TCTP is an idealized attachment surface modified to enhance cell attachment and proliferation such that damaged cells that might normally detach could stay attached and continue to contribute to the overall cell proliferation. Another
An important consideration for the soluble TA experiment is that TA was replenished during media changes such that cells were constantly exposed to full concentrations of TA in the DMEM. The ability of cancer cell lines to develop resistance to chemotherapeutic agents is well documented, however it is unlikely that the cancer cells in this study developed a resistance over such a short experiment, and under the constant stress of fresh TA-DMEM solutions.\textsuperscript{16-18} Therefore, it is likely that the decrease in proliferation of cells is a direct result of TA concentration in the DMEM. Overall the results were consistent with previous literature, and there is a lack of evidence to suggest that soluble TA would provide any preferential therapeutic effect for cancer cells over normal human body cells.

The results of this study agree with previous literature that has established that cells proliferate faster on stiffer gels (Fig. 3.5).\textsuperscript{46,47} This has been shown to be a direct result of improved integrin attachment leading to enhanced cellular signaling ability to modify the local EMC components to further induce cell proliferation.\textsuperscript{46} Although ECM composition can be mediated by local cells, so too can cell proliferation and morphology be mediated by the ECM.\textsuperscript{23} Although both melanoma cancer and fibroblast cell morphology, and genomic expression can adapt to localized ECM composition, studies have yet to investigate if fibroblast cells will more readily adapt to soft collagen gels compared to melanoma cancer cells. There was no difference in cell proliferation ratio between cell types for cells seeded onto stiff TCTP matrix and ECC gels. However there was a marked difference in day 5
proliferation ratio between cell types seeded onto soft UC gels across TA crosslinking treatments, indicating that the synergistic effect of TA and matrix stiffness may play a preferential role in targeting cancer cells on softer gels, due to the non cancer cells inherent ability to more readily adapt to softer matrices. Stiffness is speculated as the main driver of the differences detected in cell proliferation as the quantification experiment was able to show that there was no significant difference in the amount of incorporated TA between UC and ECC gels. However, another potential factor could be enzymatic inhibition of selective virulent factors only secreted by cancer cells.\textsuperscript{36}

A study by Koide was able to show that TA crosslinked type I collagen was resistant to collagenase, a matrix metalloproteinase (MMP) responsible for breaking peptide bonds in collagen molecules.\textsuperscript{44} MMPs are considered a virulence factor meaning they are secreted by pathogens, such as tumor cells, to promote colonization of the host. A study by Yamanishi et al. showed that melanoma spreading into the basal lamina of the skin requires collagenolytic activity to disrupt the collagen barrier, and so it is important to identify counter measures to combat the activity of MMPs.\textsuperscript{48} Using TA as a counter measure to MMP collagen degradation, as well as a combative treatment against cell proliferation, could improve the applicability of TA crosslinked UC gels.

UC collagen gels had larger pores compared to ECC gels indicating that they may more readily degrade and release TA molecules. Quantification of TA
release profile into media from UC and ECC gels were not detectable indicating that negligible amount of TA was released from the gels. These results also suggest that cells primary exposure to the TA may be through direct contact with the gels, further indicating that TA might have altered integrin-mediated cellular attachment to the gels. UC collagen gels also had a rougher surface topography indicating that cells may be exposed to a higher amount of surface area compared to ECC gels. Cass et al. already demonstrated a preferential tendency of TA to target breast cancer cell proliferation on soft gels, and the results of this study show a similar outcome.\textsuperscript{29} However on stiffer gels, there is no preferential effect of TA on cancerous cells. Therefore larger pore size, increased TA surface area exposure, and matrix stiffness could all play a synergistic role in targeting melanoma cancer cell proliferation over NIH 3T3 fibroblast proliferation on soft UC collagen gels.

This study was the first to explore the synergistic effects of TA and matrix stiffness on metastatic melanoma cell proliferation, and as such the focus was not on identifying the underlying molecular mechanisms through which that was accomplished. Future studies should seek to explore the mechanisms by which TA inhibits cell proliferation to better understand its preferential effect on melanoma cancer cells only on soft UC collagen gels. Furthermore, it remains to be determined how cellular uptake of soluble TA differs from bound TA uptake, and whether TA crosslinked UC collagen gels more readily give up bound TA compared to ECC collagen gels. Future studies should also attempt to conduct
mechanical testing of crosslinked and uncrosslinked UC gels. The soluble TA experiment should be expanded to include more treatments of TA-DMEM, potentially in concentrations of 10 and 20 g/L, to further investigate the presence of a preferential effect of TA on melanoma cancer cells. Finally, more ECM components, such as elastin, GAGs, and fibronectin, should be included into collagen gels to more accurately model melanoma ECM composition and investigate cellular response on stiff vs soft collagen gels.
Chapter 5
Conclusions

The major conclusions of this work are summarized below:

1. TA crosslinking induces collagen fibril merging resulting in decrease in pore size for both UC and ECC collagen gels.

2. TA crosslinking increases the strength and modulus of ECC collagen gels. Furthermore, amount of TA incorporated within both gels is comparable at all TA concentrations.

3. A375 melanoma cells showed greater spreading on ECC gels compared to UC gels indicating that matrix stiffness impacts cell morphology.

4. Alamar blue assay showed that A375 cells proliferated significantly faster on ECC (stiff) collagen gels compared to UC (soft) collagen gels.

5. Alamar blue assay showed that TA had a greater effect on A375 cell proliferation than NIH 3T3 fibroblasts on UC collagen gels. However, on ECC collagen gels, the proliferation of both cells types were equally affected by TA.

In conclusion, the results of this study suggest that gel stiffness plays an important role in tumor cell proliferation and progression. Furthermore, TA shows considerable promise to be utilized as an anticarcinogenic agent when incorporated into UC collagen gels.
Chapter 6
Future Studies

TA crosslinking improved the strength and modulus of ECC collagen gels, however it was not possible to characterize these changes for crosslinked UC collagen gels using tensile testing. Therefore it is proposed that future work seek to characterize the mechanical properties of TA crosslinked UC gels through the use of atomic force microscopy or some other nanoindentation techniques. The ability to characterize the stiffness of UC collagen gels will allow for a more thorough cross comparison of matrix stiffness across UC and ECC gel type.

Further it is encouraged that future studies seek to characterize the mechanisms through which TA disrupts melanoma cell proliferation. Although the mechanisms have been described for a leukemia cancer cell line, and some degree for breast cancer, they are not described for melanoma. Further understanding of the specific processes that are disrupted could allow for a more targeted and effective use of TA as a treatment option.

Finally we encourage the use of in vivo models to validate the findings presented from the in vitro models employed in this study. The use of murine models would more accurately model the heterogenic tumor ECM, and further develop targeted TA delivery methods. The oral administration of TA has been previously employed to mediate the effects of doxorubicin cardiotoxicity, however
this is not a site specific delivery method. It is hypothesized that the use of the TA crosslinked collagen gels as dermal patches implanted directly next to tumor cells would allow for a highly site specific TA delivery method that would, ideally, preferentially inhibit cell tumor cell proliferation compared to the surrounding non cancerous cell types.
References


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