CELL ATTACHMENT TO ZEIN SURFACES

BY

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THESIS

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ABSTRACT

Nanostructured biomaterials have been recently investigated in various biomedical applications, such as tissue engineering and circulating cancer cell retrieval. Understanding the mechanism of cell-substrate interaction is pivotal for exploiting the potential of those materials. Adhesion of cells to substrate surfaces is often mediated by tissue transglutaminase (tTG) in its role as a crosslinking enzyme.

Zein, a corn protein, has been recently studied as a biocompatible material in tissue engineering. It is a prolamin containing multiple poly-glutamine strands. Previous studies suggested that glutamine residues on zein structure are involved in NIH 3T3 cell adhesion. Considering that tTG mediates cell adhesion mainly through crosslinking between glutamine residues and lysine residues, we hypothesized that the possible function of zein glutamine residues to cell adhesion may be associated with tTG. To explore this hypothesis, a zein film was constructed with glutamine residues exposed to cells. Results confirmed that zein substrate enhanced cell spreading and adhesion. SDS-PAGE confirmed zein is a glutamine donor for tissue transglutaminase. Deposition of tTG on zein coating films further enhanced cell spreading and adhesion. Thus, the interaction between zein and 3T3 may be mediated with tTG.

Based on this result, we also hypothesized zein substrates may be useful in capturing cancer cells. Results showed that zein films enhanced adhesion of MDA-MB-231 line cells. Tissue transglutaminase treatment on zein films further enhanced cell spreading and adhesion. Cell capture increased with incubation time. At short incubation times, cell capture increased with zein content in the coating film. Cell capture yield increased linearly with tTG concentration. This study provided supporting evidence for the development of a zein platform for CTCs capture.

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Chapter I. Introduction

Biomaterials, such as polymers and ceramics, are integral components of structural systems destined for medical applications. An important challenge in medical applications of engineered biomaterials is to overcome foreign body reactions. An approach taken to minimize this effect is the coating of implants with biocompatible coatings (Rolfe, 2011). Zein, a major corn protein, has been proposed as biomaterial for tissue engineering. It can readily form film/coatings. Zein is biocompatible with several cell lines, including human liver cells and mice fibroblast (Dong, Sun, & Wang, 2004).

Circulating tumor cells (CTCs) are cancer cells originated in solid tumors then shed into the circulatory system. They are responsible for cancer metastasis. Because they possess biomarkers for cancer diagnosis and therapy, there is great interest in their retrieval and isolation from blood samples (Danila, Fleisher, & Scher, 2011). Biomaterials are currently under investigation as a platform for enhancing the capture of circulating tumor cells (L. Wang, Asghar, Demirci, & Wan, 2013).

Zein film properties are based on its unique molecular structure, which takes the shape of a rectangular prism formed by 9 or 10 nearly identical α-helix segments stacked together in an anti-parallel fashion. Helices are linked at each end by polyglutamine strands, which give the top and bottom of the molecule a markedly hydrophilic character (Matsushima, 1997). Wang et al. (2008) observed that 3T3 cells attached to the glutamine surfaces of zein molecules. Glutamine plays several important roles in cell metabolism, including providing nitrogen for protein synthesis (Newsholme, Procopio, Lima, Pithon-Curi, & Curi, 2003). The hypothesis presented here is that glutamine residues on zein structure may interact with cells and promote cell adhesion. To construct zein nanostructures with exposed glutamine residues, O_2 plasma treated glass was used to induce the hydrophilic adsorption of a zein layer, which generated an opposite glutamine rich zein surface.

In this study, NIH3T3 mouse fibroblast cells were used to investigate the potential of zein films as biomaterial coatings and MDA-MB-231, a breast cancer cell line, was used as surrogate to investigate CTC retrieval. Cell-matrix interactions are important for both applications. In this

study, cell spreading and adhesion parameters were measured to study cell-matrix interactions. The objectives of this work were:

1. To investigate NIH 3T3 cell attachment to constructed zein substrates.
2. To investigate the mechanism of cell attachment and determine whether glutamine is involved in cell-substrate interaction.
3. To investigate MDA-MD-231 cell attachment on constructed zein substrates.

Results of this study may provide preliminary evidence for the development of zein applications in tissue engineering and cancer cell retrieval.

Chapter II. Literature review

2.1 Biomaterials

2.1.1 Implants

Implants are biological or artificial materials inserted into the body. With the increasing life-expectancy and an aging population demographics increasing, there is an increased demand for medical implants. Unfortunately, the implantation procedures are often associated with tissue response continuum, which include injury, blood-material interaction, inflammation, foreign body reaction as well as fibrous encapsulation of foreign materials. The initial response to injury is blood flooding. Fibrinogen from the blood form blood clots (Ratner, 2004). Injury to the vascularized tissue during the implantation process initiates vascular and cellular inflammatory response (Anderson, 2001). Monocytes from the blood are recruited to the wound site and differentiate into macrophages, which would clear up the bacteria and dead cells. Meanwhile, endothelial and fibroblast cells are called to convert the blood clot to the granulation tissue, which is followed by remodeling of the extracellular matrix. Whether the tissue can be completely recovered depends on the degree and location of the injury (Ratner & Bryant, 2004).

However, if a foreign material is placed in the tissue, it may generate a different response, which is called foreign body reaction. Foreign body reaction is a process where the inflammatory cells migrate to the implant site to destroy the foreign material (Rolfe, 2011). It includes protein adsorption onto the material, cell attack, giant cell formation and collagen encapsulation of the material (Ratner & Bryant, 2004). If the material cannot be phagocytosed, it would eventually be encapsulated in thick fibrotic capsules and isolated from the tissues. Thus, implant materials are poorly integrated into the tissue, which contribute to the failure of may implant devices (Anderson, Rodriguez, & Chang, 2008; Rolfe, 2011).

In recent years, many biomaterials are engineered to control foreign material reactions. An optimal material is normally determined by its bulk and surface properties. Often times, it is not economically or technologically practical to change the bulk properties. Thus, surface properties are often used to enhance performance of the material (Kurella & Dahotre, 2005). In bone implants, surface treatment that can induce osseointegration would be preferred (Bauer, Schmuki, Mark, & Park, 2013). The surface characteristics that influence tissue response include surface chemistry,

morphology, size and porosity (Morais, Papadimitrakopoulos, & Burgess, 2010). Implant materials mainly consist of four systems, metals, polymers, ceramics, and composites (Kurella & Dahotre, 2005). Surface modification to implant materials include physical treatments such as blasting, etching and plasma spraying to modify surface topography and roughness, as well as chemical treatments to optimize interactions between materials and tissues. The most direct chemical treatment to implant materials is coating. Calcium phosphate, for example, is commonly used to coat metals for bone implants due to its similarity to natural components in bone. Also, surface functionalization with biomolecules has been extensively studied in recent years to convert bio-inert surfaces to bio-active ones (Bauer et al., 2013). For example, RGDs sequence found in many extracellular matrix proteins such as collagen and fibronectin have been used to coat surfaces and significantly enhanced cell adhesion onto materials surfaces (Brandley & Schnaar, 1988; Pierschbacher & Ruoslahti, 1984). In order to reduce the fibrotic response, biocompatible coatings including synthetic and natural polymers have been used to mask implant materials (Galeska et al., 2005; Geiger, Li, & Friess, 2003; Rolfe, 2011).

2.1.2 Circulating tumor cells (CTCs) retrieval

In modern societies, cancer has become a leading cause to death, only secondary to cardiovascular disease (David & Zimmerman, 2010). Cancer cell is described as uncontrolled cell proliferation form abnormal cell accumulations or tumors. At the metastatic state, cells would spread, seed other parts of the body and invade the host tissues. Cancer metastasis is a process where cancer cells from the primary tumor adapt to the microenvironment of another tissue. Cancer metastasis accounts for 90% death due to solid tumors (Gupta & Massague, 2006). In order for metastasis to happen, cancer cells must evade the evolution barriers. Metastasis involves several steps. Cancer cells first lose cellular adhesion and increased motility; then, cells enter the circulatory system; and last, cells colonize a specific tissue (Chambers, Groom, & MacDonald, 2002; Fidler, 2003; Gupta & Massague, 2006). Normally, only a small amount of circulating tumor cells (CTCs) are involved in seeding the metastasis colonies (Cristofanilli, 2006). A specific type of cancer normally has a selection of metastasis sites. This metastasis pattern was proposed to be associated to compatibility with the target sites. Breast cancer metastasis would frequently occur in lung, liver, brain and bone. Though most of these tissues are not directly connected to breast tissue (Fidler, 2003; Gupta & Massague, 2006).

Clinical studies showed that detection of CTCs allows for the sub-classification of breast cancer. Solid tumors contain heterogeneous cellular types and only cancer stem cells are tumorigenic. These cells are responsible for cancer propagation and present major challenges in cancer treatment (Balic, Lin, Williams, Datar, & Cote, 2012; Wicha, Liu, & Dontu, 2006). It has been demonstrated that a subpopulation of CTCs has the phenotype of stem cell progenitor (Theodoropoulos et al., 2010). CTC provides important diagnostic information in cancer patient (Alix-Panabieѐres & Pantel, 2013; Kang & Pantel, 2013; Van De Stolpe, Pantel, Sleijfer, Terstappen, & Den Toonder, 2011).

An important step to enhance clinical diagnosis, would be the isolation of CTCs from patients' blood. One of the biggest challenges for CTCs detection is that their percentage among blood components is very low, it is estimated at one CTC in 10^7 white blood cells per milliliter of blood (Krebs et al., 2014). Thus, various techniques aimed at enriching and detecting CTCs have been developed (Balic et al., 2012). Up until now, immunomagnetic approach has been most successful. The epithelial cell adhesion molecule (EpCAM) is frequently used for separating epithelial CTCs from blood cells. Magnetic beads with functionalized groups were developed to bind to CTCs, so blood cells could be separated. Recently, a microchip platform was also designed to capture cancer cells. Microchips with EpCAM antibody coating demonstrated effective cell capture. (Maheswaran et al., 2008; Nagrath et al., 2007). However, the EpCAM based system is not sensitive for cells that undergo epithelial to mesenchymal transition (EMT), thus assays based on this marker underestimate CTCs number in late metastatic patients (Gorges et al., 2012; Krebs et al., 2014).

Another approach is the use of nanostructured materials. Nanostructure increases cell-substrate interactions and thus enhances the efficiency of cell capture (Fischer et al., 2009; S. Wang et al., 2011) He et al. (2013) developed a TiO_2 nanofilm coated with anti-EpCAM antibody. The substrate successfully captured CTCs from the peripheral blood sample of a cancer patient. Mitchell et al. (2015) developed halloysite nanotubes (HNTs) coated with selectin protein. This substrate showed increased cell adhesion in flowing conditions as well as facilitated cell capture without the presence of antibodies. Capture strength was also studied. Results showed that when shear stress of 180 dyn cm^{-2} was applied, 50% cells could be detached. Recent interest in CTCs isolation and detection also led to the proposal of other cost effective methods.

2.2 Zein as biomaterial

Zein is the storage protein in maize that is soluble in alcoholic solutions (Larkins, Pedersen, Marks, & Wilson, 1984). Zein consists of four sub-categories, namely α-, β-, γ- and δ − zein. α-zein, the most abundant fraction, includes bands of relative molecular weight of 19kDa and 22kDa (Shewry & Tatham, 1990). β-zein, second most abundant protein, has a molecular weight of 17kDa (A. Esen, 1987). Gamma zein includes two components of 18kDa and 27kDa, which together account for 10-15% of zein protein (Esen, 1990). α-zein forms oligomers, but most of them are soluble in aqueous ethanol. However, β-zein and γ- zein are only extractable in the presence of a reducing agent. Thus, they are considered as large disulfide-linked polymers (A. Esen, 1987). δ − zein is minor fraction of zein with a size of 10kDa and also only extractable in the presence of reducing agents (a Esen, 1990; Shewry & Tatham, 1990). In terms of amino acid composition, the four groups share the similarities; they are rich in glutamine and proline, but low in lysine and tryptophan. β-zein and γ- zein are higher in methionine than α-zein and β- zein, while cysteine and histidine are both higher in γ-zein (Shewry & Tatham, 1990).

2.2.1 α-zein amino acid sequence

The complete amino acid sequence of α-19 zein was first deduced from nucleotide sequence in 1981(Geraghty, Peifer, Rubenstein, & Messing, 1981). They observed a short signal peptide attached to the N-terminal and 7-8 tandem repeats structure. Dipeptide such as Ala-Ala, Leu-Leu and Gln-Gln occurred respectively 7, 8 and 14 times. Later on, Pedersen et al. (1982) detected 7-9 repeat sequences consisting of approximately 20 amino acids. It was also found that the glutamine position was often more stable in these repeats. In that study, the signal peptide and the initial 37 and last 8 amino acids were unique. In between, repeating structures shared consensus characteristics. They start with glutamine and always follow the "Polar-hydrophobic-polar-hydrophobic-polar" pattern. The consensus repeat sequence was also reported by other groups (Heidecker, Chaudhuri, & Messing, 1991; Thompson, Siemieniak, Sieu, Slightom, & Larkins, 1992). It was suggested the repeat structure facilitated the packing of zein proteins within protein bodies (Argos, Pedersen, Marks, & Larkins, 1982). Figure 2.1 shows the consensus amino acid sequence of α-19 zein and α-22 zein (Tatham et al., 1993). **Table 2.1** shows the percent amino composition of α-19 zein.

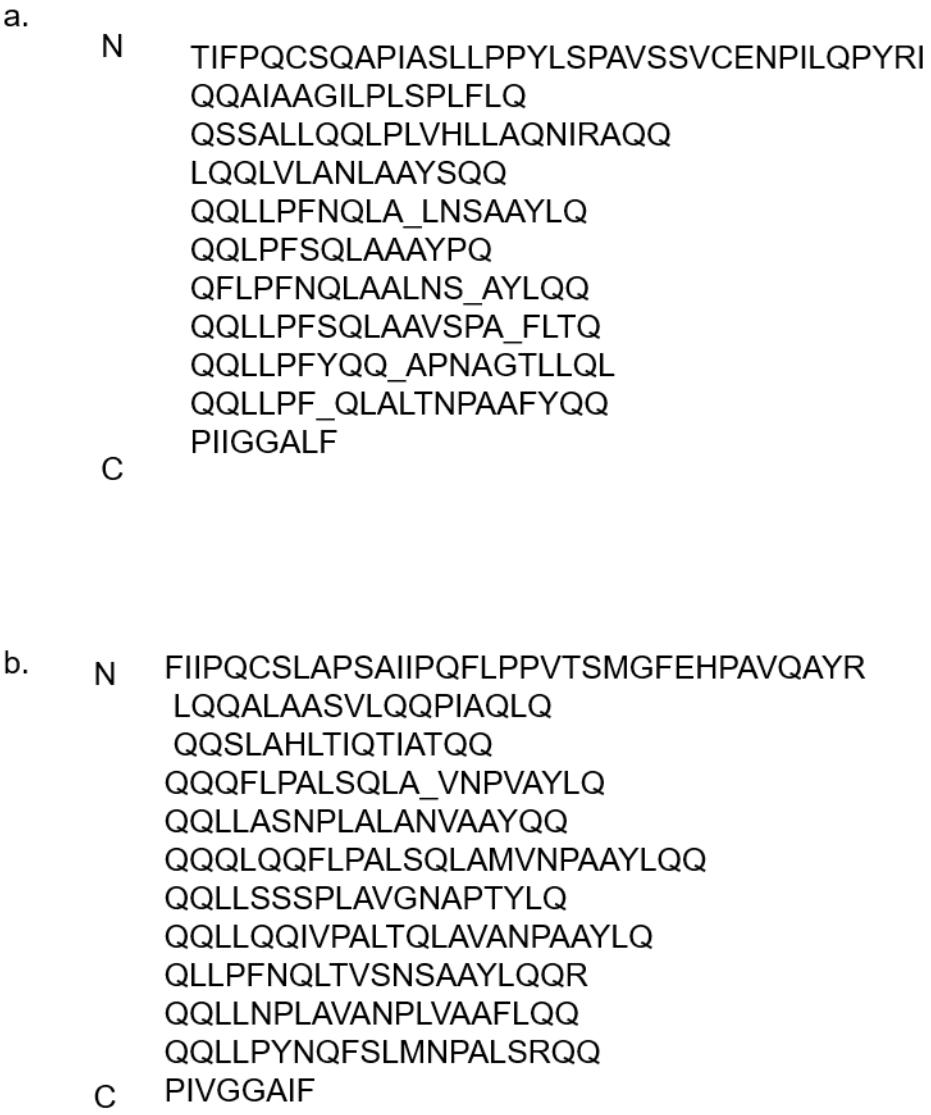

Figure 2.1 Consensus amino acid sequence in Z19 (a) and Z22 (b) (Tatham et al., 1993)

Table 2.1 Amino composition of α-19 zein.*

Amino acid	# residues	Amino acid	# residues	Amino acid	# residues
Leucine	48	Asparagine	11	Glycine	2
Glutamine	43	Threonine	8	Histidine	1
Alanine	35	Tyrosine	8	Lysine	1
Proline	22	Valine	7	Glutamic acid	1
Serine	19	Methionine	3	Aspartic acid	1
Phenylalanine	13	Arginine	3	Tryptophan	0
Isoleucine	11	Cysteine	3	**Total**	**240**

* Coleman & Larkin (1999); Nonthanum (2013)

2.2.2 Zein structural model

Several structural models have been proposed for α-zein. According to amino acid sequencing, α-19 and α-22 zein have, respectively, 9 and 10 repeated helices. The consensus sequence contains three polar segments, each of them consists of two polar amino acids. The turn region between helices is three to four amino acids, thus Argos et al. (1982) proposed that the nine anti-parallel helices each could be arranged in a cylinder structure and the polar segments of these helices are connected through hydrogen bonding or intermolecular contacts.

Matsushima et al. (1997) modified the previous model to account for the asymmetric shape of α-zein molecule shown by SAXS analysis. Similar to the previous model, the tandem repeat units were α-helices connected by glutamine turns. But, in this model, the 9 or 10 α-helices stack together the direction perpendicular to the helix axis, forming a rectangular prism 16nm x

1.2nm x 4.6nm. The top and bottom of the prism contain glutamine rich turns or loops, rendering the top surfaces hydrophilic and the lateral sides hydrophobic.

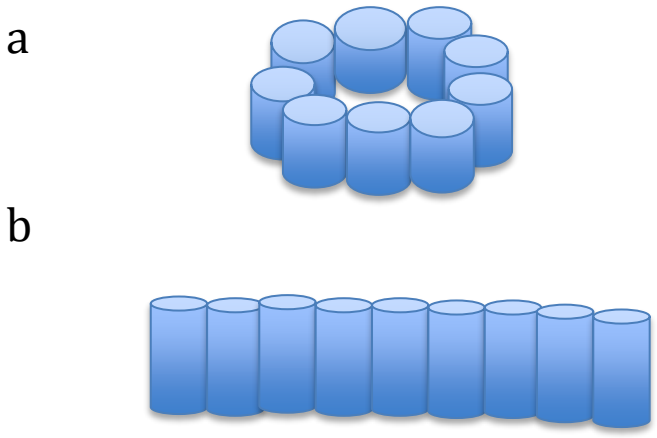

Figure 2.2 Zein structure model a) Argos et al. (1982) b) Matsushima et al. (1997)

2.2.3 Surface control orientation

Wang et al. (2004) studied the adsorption of zein on either hydrophilic 11-mercaptoundecanoic acid or hydrophobic 1-octanethiol monolayers self-assembled on glass surfaces. It was found that the initial adsorption of zein was higher on hydrophilic monolayer. More importantly, the monolayer value was larger on 11-mercaptoundecanoic acid, indicating that zein may have different orientations depending on the characteristics. In another study (Q. Wang et al., 2008), zein was adsorbed on 16-mercaptohexadecanoic acid hydrophilic patterns. NIH 3T3 showed cell growth limited to the patterned region. Their study demonstrated that zein orientation was affected by the hydrophilic/hydrophobic character of the substrate.

Surface plasma modification is a technique used to modify surface properties of biomaterials and consequently, their performance. The function of biomaterials is largely dependent on their surface chemistry and mechanical properties. But materials with good mechanical properties often lack good biocompatibility. Plasma modification can potentially enhance surface characteristics of biomaterials and render them more responsive to cells. Plasma is ionized gas composed of molecular, ionic, radical and atomic species (Chu, Chen, Wang, & Huang, 2002). It contains equal amounts of positive and negative charges (Liston, 1989). O_2 plasma treated glass increased surface

roughness and reactivity. Meanwhile, it also generated very hydrophilic surfaces with contact angles below 2 degrees (Alam, Howlader, & Deen, 2014). O_2 plasma treated glass demonstrated enhancement of PDMS binding to glass (Xiong, Chen, & Zhou, 2014). Hexamethyldisiloxane treated with O_2 plasma showed increased –OH functional groups, which are associated with increased hydrophilicity and the concomitant absorbance of simvastatin acid onto the surface (Yoshinari et al., 2006). Similarly, Wei et al. (2007) observed that O_2 plasma treated hexamethyldisiloxane increased the number of fibroblast cells adhered in the initial stage. Hydrogen free diamond-like carbon also increased Chinese Hamster Ovary cell adhesion (Araujo, Teixeira, da Silva, Salvadori, & Salvadori, 2014).

2.2.4 Biocompatibility

Zein films were used as substrates to grow liver HL-7702 cells and mice NIH3T3 showing good biocompatibility (Dong et al., 2004). Also, zein degradation products also showed better biocompatibility than Corning plate to human umbilical veins endothelial cells (H. J. Wang, Lin, Liu, Sheng, & Wang, 2005). For microfluidic biosensor application purposes, improvement on film transparency was obtained by heating films to121ºC at 100% RH and 103.4 kPa. Treated films showed no decease in cytocompatibility (Han, Xu, Lu, & Wang, 2014).

In tissue engineering research, scaffold should provide good mechanical properties to support cell attachment (Chan & Leong, 2008). Citric acid was used as cross-linker to enhance fiber stability in phosphate-buffered saline. Cross-linked nanofibers adhesion, spreading, and proliferation for fibroblast cells (Jiang, Reddy, & Yang, 2010). Cell differentiation was enhanced in MG63 cells when zein and hydroxyapatite were incorporated into poly(epsilon-caprolactone) (Salerno et al., 2012).

2.3. Cell behavior

2.3.1 Test cells

NIH 3T3

NIH 3T3 is an immortal mouse fibroblast cell. Fibroblasts represents a large family of cells in connective tissue and only myofibroblast in this family was formally defined. It is often identified by its stellate, elongated and branchy structure with rough endoplasmic reticulum. In wound

healing, keratinocytes migrate and fill the cut by cell proliferation. All the while, they secrete growth factors, including transforming growth factor-beta (TGF-beta) and epidermal growth factor (EGF) that stimulate angiogenesis and fibrogenesis. The angiogenesis process forms tubular structures that allow transport of nutrients (Darby & Hewitson, 2007). Fibroblasts produce high amounts of the extracellular matrix (ECM) proteins, collagen and fibronectin, which support other cells in the development of tissue structure (Kisseleva & Brenner, 2008; Bainbridge, 2013).

MDA-MB-231

Breast cancer is a common cancer type among women. The majority of deaths from breast cancer is due to cancer metastasis (Cristofanilli, 2006). Various breast cancer cell lines *in vitro* models have been developed to study this cancer. The metastatic breast cancer cell line MDA-MB-231 was isolated from pleural effusions of a breast cancer patient in 1973 (Cailleau, Young, Olivé, & Reeves, 1974). It is characterized for lacking in estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2).

2.3.2 Cell adhesion and spreading

The development of biosensors for monitoring cell processes requires enhanced cell adhesion to the device surfaces involved. Cell adhesion and spreading also participate in cellular behavior, including cell differentiation, cell proliferation, and migration. In serum supplemented medium, cell adhesion is mediated by extracellular proteins. Bioactive proteins adhere to the substrate surface based on their physicochemical properties and adsorption patterns impact cellular response (Wilson, Clegg, Leavesley, & Pearcy, 2005). Fibronectin and vitronectin are two major adhesive proteins in ECM. Fibronectin is excreted by the cell into the ECM. It is also provided in serum supplemented cell culture medium. For fibronectin deleted mouse embryonic cells, their growth was positively associated with fibronectin supplied in the medium, which suggested the role of fibronectin in cell growth (Sottile, Hocking, & Swiatek, 1998). Differences in adsorptive patterns of adhesion proteins on the substrate surface can impact cell adhesion and spreading. For example, serum albumin coating was observed to decreased fibroblast adhesion (Tamada & Ikada, 1993).

Integrins are trans-membrane adhesion receptors. They act as a bridge for cell-ECM interactions (Hynes, 2002). Integrin recognizes the Arg-Gly-Asp (RGD) sequence on various matrix proteins, including fibronectin and vitronectin (Barczyk, Carracedo, & Gullberg, 2010; Hynes, 2002).

Integrin activity is controlled by signals from within the cell via conformational changes and by signals from outside of the cell (Takada, Ye, & Simon, 2007).

In addition to adhesion proteins, surface hydrophobicity, surface charge, roughness, stiffness and topography also affect cell adhesion and spreading (Chang & Wang, 2011). For example, when pore size increased to larger than 4nm, cell appeared round in shape (Kim, Kino-Oka, Kawase, Yagi, & Taya, 2007). When Fetal Bovine Serum (FBS) is used as serum source, fibronectin is the dominant adsorption protein on hydrophilic surfaces, but vitronectin takes the role on hydrophobic protein (J. Wei et al., 2009).

2.3.3 Effect of transglutaminase

Transglutaminase (Tgase) is a Ca2+ dependent enzyme that mediates crosslinking between glutamine residues of one polypeptide and lysine residues of another peptide (Laszlo Lorand & Graham, 2003). Six isoenzymes has been identified and characterized. Factor XIII is a fibrin stabilizing factor that participates in wound healing. Type 1 Tgase is a Keratinocyte Tgase involved in the differentiation process of Keratinocytes (Griffin, Casadio, & Bergamini, 2002). Type 2 Tgase is also called tissue transglutaminase (tTG). It is excreted by many cell types including 3T3 to the cell surface and extracellular matrix (Cai, Ben, & De Luca, 1991; Martinez, Chalupowicz, Roush, Sheth, & Barsigian, 1994; Upchurch, Conway, Patterson, & Maxwell, 1991). Type 3 Tgase is called Epidermal Tgase and is also involved in Keratinocyte differentiation processes (Griffin et al., 2002). Type 4 and Type 5 Tgase are respectively associated with reproductive functions in rodents and epidermal differentiation (Griffin et al., 2002). This thesis work focus on Type 2 Tgase.

tTG is composed of four highly conserved domains, which include N-terminal beta-sandwich (with fibronectin and integrin binding site), core domain (contains both the regulatory site and catalytic site) and C-terminal beta-barrels 1 and 2 (Fesus, 2002; Griffin et al., 2002). It is a multi-functional enzyme that can deamidate protein, incorporate amine into protein and mediate protein cross-linking.

Functions of tTG in cells

tTG is expressed at high levels in some cell types, such as endothelial and smooth muscle cells. However, in other cell types, it is only induced by specific factors, such as retinoic acid and TGF-

beta. tTG at membrane locations can interact with cytoskeleton components under the activation of calcium (Fesus, 2002). Earlier, tTG was reported to be pro-apoptotic. It was found to be induced in programmed cell death (Fesus, Thomazy, & Falus, 1987). In the human neuroblastoma cells, overexpression of tTG was associated with increased vulnerability of the cells to apoptosis (Melino et al., 1994). The mechanism of tTG in cell apoptosis was proposed to be associated with crosslinking of intracellular proteins when tTG was switched to crosslinking mode by increased calcium levels at the late stage of apoptosis (Fesus, 2002; Lesort, Tucholski, Ml, & Gvw, 2000; Nemes et al, 1997; Oliverio et al., 1997). However, it was showed later that the increase of tTG and apoptosis do not always overlap. For example, retinoic acid induced tTG activity protected cells from apoptosis (Antonyak, Boehm, & Cerione, 2002). tTG was reported to protect cells from excitotoxic stress in neural like SH-SY5Y cells (Caccamo, 2013)). When glioblastomas cells were treated with tTG inhibitor monodansylcadeverine, cells were prone to death and more sensitive to chemotherapy (Yuan, 2005)

Functions of tTG in extracellular matrix

tTG s expressed inside the cell and excreted into the extracellular matrix. Evidence suggests that tTG-mediated crosslinking plays an important role in enhancing cell adhesion and spreading (Jones, 1997, Chau, 2005, Belkin, 2005)). tTG in human retinal pigment epithelial (RPE) cells was associated with increased cell adhesion and migration (Priglinger, 2004). tTG mediate cell adhesion by its interaction with integrin and matrix proteins such as fibronectin. A model was previously described in terms of the relationship between integrin, tTG and matrix proteins. In this model, tTG improved cell adhesion either by acting as a bridge between integrin and matrix protein or by formation of large complexes with the three tied to each other (Akimov, 2000)). The crosslinking reaction catalyzed by TG2 is depicted in Figure 2.3. When tTG was knocked down in human umbilical endothelial cell, cell spreading and adhesion decreased correspondingly (Jones et al., 1997).

A number of tTG substrates have been identified. Although common glutamine reactive residues cannot be defined yet, contiguous glutamines are found to be common features of several natural and artificial tTG substrates (Csosz et al., 2008; Kahlem, Terré, Green, & Djian, 1996)

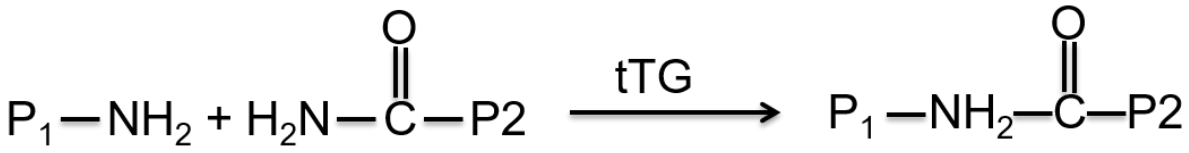

Figure 2.3 tTG catalyzed crosslinking reaction.

2.3.4 Role of glutamine in cell metabolism

Role of glutamine in cell metabolism in normal cells

Glutamine belongs to non-essential amino acid. Its primary function is generally attributed to maintaining the balance of nitrogen supply in different tissues. But, during periods of stress, such as disease, it becomes essential. In human diploid fibroblasts, glutamine utilization increased when glucose levels decreased, glutamine oxidation accounted for 30% of cell energy needs (Zielke, Ozand, Tildon, Sevdalian, & Cornblath, 1978). In 3T3 fibroblast, glutamine supplementation can even counteract growth inhibition inflicted by serum deprivation (Zetterberg & Engström, 1981). Glutamine is associated with counteracting cell stress. It is the only indispensable amino acid in heat induction of heat shock protein 25, an adaptive mechanism for cell survival in front of pathophysiological stress (Phanvijhitsiri, Musch, Ropeleski, & Chang, 2006). Glutamine also contributes to cell adhesion through the synthesis of related complex carbohydrates (Oppenheimer, 1973). Glutamine stimulated expression of collagen gene in a dose-dependent manner (Bellon, Chaqour, Wegrowski, Monboisse, & Borel, 1995). But, for some cell lines, such as MDCK, CHO-K1 and BHK-21, glutamine free medium supplemented with pyruvate does not cause drastic differences in terms of cell metabolism (Crook, Daniels, Smith, & Mcclain, 1993).

Role of glutamine in cell metabolism in cancer cells

Cancer cells have an appetite for glutamine due to their rapid growth. In fact, glutamine has been recognized as an important cancer cell nutrient. In HeLa cells, when glucose is the sugar source, more than 50% of the cell energy comes from aerobic oxidation of glutamine, while when fructose or galactose is the sugar source, more than 98% of cell energy is supplied from glutamine (DeBerardinis & Cheng, 2010; Reitzer & Wice, 1979). The glutamine consumption in transformed

cells exceeded the demand for nucleotide synthesis and amino acid pool (DeBerardinis et al., 2007).

Tumor cells express large amounts of glutaminase for using in glutamine metabolism. This enzyme has been demonstrated to be related to tumor growth rate (Knox, Linder, & Friedefi, 1970; Linder-horowitz et al., 1969). Glutamine is the major nitrogen donor for the build-up of purine and pyrimidine, the precursor nucleotides for cell proliferation. It also provides the amino group for fructose-6-phosphate, forming glucosamine-6-phosphate, essential in glycosylation reactions (DeBerardinis & Cheng, 2010; Donadio et al., 2008).

Chapter III. Enhanced 3T3 cell adhesion and spreading may be associated with tissue transglutaminase*

3.1 Abstract

Zein is a biocompatible corn protein potentially useful in the development of biomaterials. In this study, the deposition of zein on oxygen plasma treated glass cover slips significantly enhanced cell spreading and viability. The mechanism for cellular response to zein coated surfaces was thought to involve the polyglutamine peptides on the zein structure. We hypothesized that zein was a substrate for tissue transglutaminase (tTG), an extracellular enzyme involved in cell-surface interactions. SDS-PAGE results suggested an interaction between zein and tTG, where zein was the glutamine donor. Cross-linking between zein and tTG may be the first step in successful cell adhesion and spreading.

3.2 Introduction

Knowledge of the interaction between cells and substrate surface is critical for developing biomedical devices. Surface architecture influences various physiological behaviors, including cell adhesion, spreading, proliferation, migration and apoptosis. Cell adhesion in serum-supplemented medium is mediated by adhesion proteins, such as fibronectin and vitronectin (El-Ghannam, Ducheyne, & Shapiro, 1999; Underwood & Bennett, 1989; Wilson et al., 2005). Deletion of these proteins is often associated with loss of cell adhesion (Kulkarni & McCulloch, 1994; Underwood & Bennett, 1989). Bioactive proteins adhere to surfaces based on their physicochemical properties and their adsorption patterns impact cellular response (Wilson et al., 2005).. Cell spreading is a fundamental process that follows cell adhesion. It involves reorganization of the cytoskeleton and an increase in contact area with the substrate (Wilson et al., 2005). When cells adhere to surfaces but show no spreading, cell apoptosis is increased (C. S. Chen, Mrksich, Huang, Whitesides, & Ingber, 1998). Both, interaction with the surface and development of cell morphology, influence subsequent cellular behavior, such as cell proliferation.

Tissue transglutaminase type II (tTG) is a widely distributed enzyme with pleiotropic functions, including protein deamidation, incorporation of amine into protein and protein cross-linking (Griffin et al., 2002). The enzyme is expressed by various cell types including NIH 3T3 and excreted to the cell surface and extracellular matrix (ECM) (Upchurch, Conway, Patterson, &

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Maxwell, 1991; Cai & Ben, 1991; Martinez et al., 1994). Among the various functions of tTG, cross-linking has attracted attention due to its significant biological consequences. Basically, tTG mediates the cross-linking between glutamine residues on one polypeptide and the lysine residues of a second polypeptide (Laszlo Lorand & Graham, 2003). Ample evidence indicates that tTG mediated cross-linking plays a crucial role in enhancing cell adhesion and spreading (Belkin et al., 2005; Chau et al., 2005; Jones et al., 1997) It was proposed that tTG improved cell adhesion either by acting as a bridge between integrin and fibronectin or by formation of large complexes with the three tied to each other (Akimov, Krylov, Fleischmana, & Belkin, 2000). Examples of tTG substrates include fibronectin and collagen (L Lorand, Dailey, & Turner, 1988; Turner & Lorand, 1989). Although consensus glutamine reactive residues of tTG substrate cannot be defined yet, contiguous glutamines are found to be one common feature of some natural and artificial tTG substrates (Csosz et al., 2008; Kahlem, Terré, Green, & Djian, 1996) . Also, the continuous glutamine stretches need to be accessible and away from discouraging residues (Coussons, Price, Kelly, Smith, & Sawyer, 1992).

Recently, plant based proteins such as zein and soy protein are reported as potential alternative materials for tissue engineering applications due to their biodegradability, availability and low immunogenicity (Reddy & Yang, 2011). Besides, the amino and carboxyl groups in their structures enable them to adapt to different pH environments *in vivo*, making them suitable for drug delivery applications (L. Chen, Remondetto, & Subirade, 2006; Reddy & Yang, 2011). Specifically, zein has been applied in food and medical fields in the forms of film, fibers and nanoparticles (Reddy & Yang, 2011). Zein films demonstrated better biocompatibility than Corning plates with human umbilical veins endothelial cells (H. J. Wang et al., 2005). Plasmid DNA encapsulated in zein spheres was protected from degradation by DNase I and was steadily released over more than 7 days (Regier, Taylor, Borcyk, Yang, & Pannier, 2012). Additionally, hollow zein spheres fabricated from phase separation were applied to increase drug loading capacity (Xu, Jiang, Reddy, & Yang, 2011).

Interestingly, glutamine is an abundant amino acid at the zein surface, forming polyglutamine strands (Argos et al., 1982; Coleman & Larkins, 1999). Zein primary structure consists of signal sequence, N-terminal sequence, C-terminal sequence and 9-10 repeat sequences in the middle, each containing 14-25 amino acid residues (Argos et al., 1982). The tandem repeating units form

α-helices arranged in anti-parallel stacks. The neighboring helices are connected with glutamine turns (Argos et al., 1982; Matsushima, 1997). Figure 3.1 illustrates the prismatic structure of α-zein. The long dimension of the prism is 16nm, while the other two dimensions are 1.2nm and 4.6nm, respectively (Matsushima, 1997). In this conformation, polyglutamine turns located at the top and bottom of the prism, form a hydrophilic surface, while the front and back, formed by the stacked helix surfaces, are hydrophobic. Due to the existence of polyglutamine turns in the zein structure and the fact that some of them are exposed according to solvent accessibility predictions (Li, Xia, Shi, & Huang, 2011), it is not unreasonable to investigate whether zein could be a possible substrate of tTG.

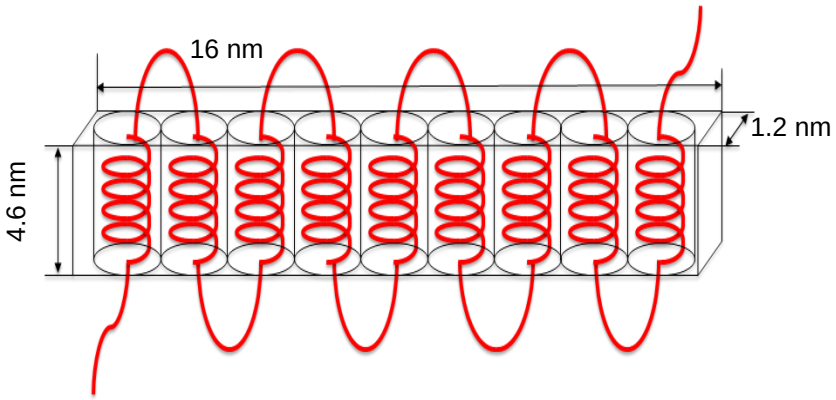

Figure 3.1 α-Zein structural model (adapted from Matsushima et al. (1997)).

In this study, we report that zein is a substrate for tTG, which may be a mechanism for enhanced cell spreading and viability on zein films for 3T3 seeded in either serum supplemented or serum depleted medium.

3.3 Materials and methods

3.3.1 Surface coating

Glass cover slips (VWR, Rednor, PA), 5 or 18 mm in diameter, were rinsed with isopropanol and placed in 12 or 96 well micro plates. Plates were exposed to oxygen plasma treatment (Diener Electronic PICO, Lomazzo, Italy) at power level of 100W and chamber pressure of 0.8-1 mbar, for 1 min. Coatings were prepared by dissolving 0.25mg/ml (Z0.25), 0.5mg/ml (Z0.5), 2mg/ml (Z2), and 10mg/ml (Z10) zein (Showa, Sangyo, Japan) in 75% isopropanol with stirring (on stir plate for 30min), pH adjusted to 4.0±0.5 with Chloroacetic acid (ACROS, New Jersey), according to Wang et al (Q. Wang et al., 2008), and then pipetted into micro plates (2ml for 12 well plate

and 200ul for 96 well plate). After 1h incubation at room temperature, the solution was discarded. Wells were rinsed with Milli Q water and dried. Oxygen plasma treated cover slips without zein coating served as control surfaces (Z0).

For the tTG treatment group, tTG solution was prepared fresh by dissolving 0.15mg/ml tissue transglutaminase (Sigma, St Louis, MO) in tTG buffer containing 1mM Dithiothreitol (Sigma, St Louis, MO), 3mM $CaCl_2$ (Fisher Scientific, Waltham, MA) and 10mM Tris-HCl pH 8.3. Zein coated coverslips (5mm diameter) prepared as above (Z0.5) were further incubated in tTG solution for 2h at 37˚C in a 5% CO_2 incubator. Wells were washed, dried and UV sterilized for 15min before cell seeding. A zein coated cover slip treated with tTG buffer was used as control (tTG_0).

3.3.2 Cell culture

NIH 3T3 cell line was cultured in DMEM medium (Fisher-Mediatech, Manassas, VA) supplemented with 10% bovine calf serum (Life Tech, Carlsbad, CA) and 1% penicillin/streptomycin (Fisher-Mediatech, Manassas, VA) at 37˚C in a humidified 5%, CO_2 incubator. Cell line was used in low passage number.

3.3.3 Cell spreading assay

NIH 3T3 cells were trypsinized, collected in serum supplemented or serum deprived medium and seeded into 96 or 12 well plate at a density of 10^4 or 2×10^4 cells/ml (100ul or 2ml). Cell morphology images were taken with an inverted microscope (Olympus IX81, Tokyo, Japan). More than 40 cells were selected for quantification. Cell outlines were manually traced by Image J software (National Institute of Health, Bethesda, MD). Cell morphology parameters including area, perimeter and circularity were automatically measured after tracing. Circularity was calculated as 4π (area/perimeter^2). A circularity value of 1 means a perfect circle. For tTG treated substrates, cells were seeded in serum supplemented or serum deprived medium at a density of 10^4 or 10^5 cells/ml (100ul, 96 well plate).

3.3.4 Cell adhesion assay

At confluency, cells were trypsinized and collected in serum supplemented or serum deprived medium and seeded into 96 well plate at density of 10^4 or 10^5 cells/ml (100ul). The viability of attached cells was quantitatively determined with Cell Counting Kit 8 (Dojindo, Rockwille, MD),

according to the manufacturer's instructions. The medium was discarded after incubation and wells were washed with 1×PBS before fresh medium was pipetted in. Cell Counting Kit 8 reagent (10ul) was added into each well and incubated for 2h at 37˚C and 5%, CO_2. Absorbance was measured with a micro plate reader (BioTek Instruments, Winooski, VT) at 450nm.

3.3.5 SDS-PAGE

Tissue transglutaminase was dissolved in tTG buffer by gentle pipetting and then combined with zein dissolved in 75% isopropanol. After 2h incubation at 37˚C and 5% CO_2, the mixture was diluted 1:1 with 2× Laemmli sample buffer (Bio Rad, Hercules, California). Proteins were boiled for 5min and separated using 10-20% Tris-HCl ready gel (Bio Rad, Hercules, California). Precision plus Protein Dual Color served as molecular weight marker (Bio Rad, Hercules, California). Gel was stained with Simply Blue (Invitrogen, Grand Island, NY) for 24h and de-stained with DI water. Image was captured with GL 4000 Pro Imaging System (Carestream Health, Rochester, NY).

3.3.6 Dansylcadaverine (MDC) incorporation

3mM MDC (Sigma, St Louis, MO) was added to tTG buffer before mixed with tTG and zein solution. The mixture was placed in 37˚C, 5% CO_2 incubator for 2h. After dilution with 2×Lammli sample buffer, proteins were separated by SDS-PAGE. Gel image was taken under both UV light and white light and then merged.

3.3.7 SEM

Zein coated cover slips were mounted on specimen stubs and spin coated with a thin layer of 60% gold and 40% Palladium (20mA, 40s, around 10nm) with a sputter-coater Emitech K575 (Emitech, Ashford, UK). Images were taken with a Hitachi S4700 High-resolution scanning electron microscope (Hitachi, San Jose, CA).

3.3.8 AFM

The zein substrate was prepared and dried. Images were taken using an Asylum MFP-3D-SA atomic force microscope (Asylum Research, Santa Barbara, CA) in intermittent-contact mode in

air. Samples were scanned at 1 Hz at a 90 degree scan angle using a BudgetSensors Tap300Al-G tip (Ted Pella, Redding, CA) with nominal resonant frequency of 300 kHz and nominal spring constant 40 N/m.

3.3.9 Statistical analysis

Differences between datasets were analyzed using Student's *t-test* and Two-way ANOVA (SAS institute, Cary, NC).

3.4 Results and discussion

3.4.1 Surface characterization

AFM image of zein coated glass cover slip (Z2) showed a uniform topography (Figure 3.2a) indicating the formation of a zein film. White streaky bits were considered to be loose particles on the surface. An SEM image of a Z10 surface (Figure 3.2b) shows the formation of scattered zein spheres on top of the films. Film formation was confirmed with Nile blue staining (see Appendix Figure B.2). Wang et al (Wang, Wang, Geil, & Padua, 2004) showed that zein adsorption to hydrophilic self-assembled monolayers formed by 11-mercaptoundecanoic acid (COOH (CH2)10SH) increased rapidly at the initial stage, but then adsorption rate decreased and reached a plateau. It is likely that in our study, adsorption changed over time showing a similar pattern.

3.4.2 Effect of zein coatings on cell spreading and viability

It was reported that adhesion and spreading of 3T3 cells on zein hydrophilic films as opposed to zein on hydrophobic surfaces (Wang, Xian, Li, Liu, & Padua, 2008). They suggested that polyglutamine strands in the zein sequence may be related to cell adhesion. In the present study, oxygen plasma treatment was applied to glass cover slips to increase their hydrophilic character. This would favor adsorption of the glutamine rich surfaces of zein (see Figure 3.1) making an oriented layer of zein molecules with the opposite glutamine rich surface accessible to adhering cells.

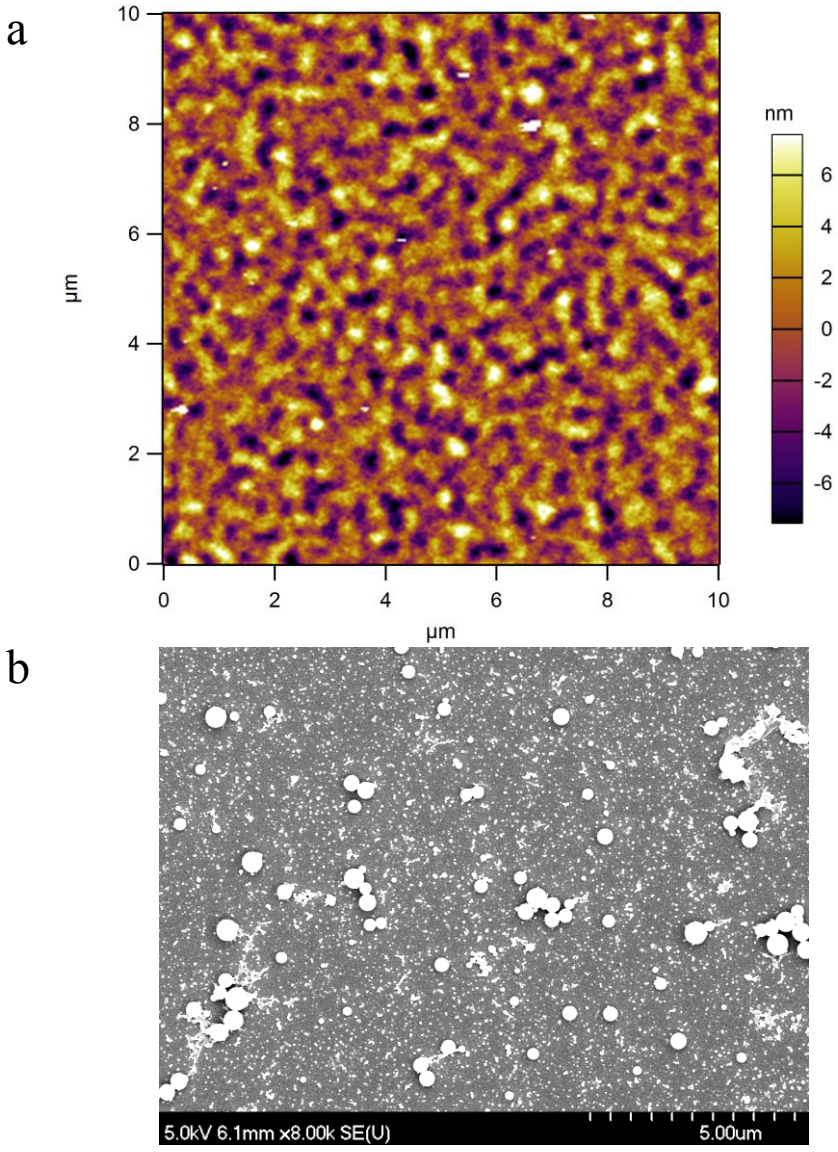

Figure 3.2 (a) AFM image of zein coating Z2 and (b) SEM image of zein coating Z10.

Cell spreading parameters on Table 1 show an increase in area and perimeter, and lower circularity for cells seeded on zein coated surfaces Z0.25 with respect to Z0, when using serum supplemented medium. Serum provide cell adhesion proteins, such as fibronectin, which enhance cell spreading

(Horbett & Schway, 1988). Elter et al. (2012) reported an increased adsorption of the cell adhesion protein fibronectin on nanostructured surfaces compared to smooth surfaces. The nanostructured topography of zein surfaces, observed by AFM (Figure 3.2a), may have enhanced adsorption of cell adhesion proteins. Higher amount of zein on coatings (Z0.5 – Z10) did not further increase cell spreading. It is possible that in this range of zein content on coatings, overall nanostructure remains similar, resulting in similar adsorption of cell adhesion proteins and thus cell spreading on Z0.25 to Z10 substrates.

Cell spreading parameters for 3T3 cells in serum deprived medium are also shown on Table 3.1. It was (Kulkarni & McCulloch, 1994) reported that serum deprivation induced apoptosis and loss of cell attachment. In this study, serum deprivation induced cell rounding and death on control surface, Z0 (Figure 3.3 a). However, serum deprived 3T3 cells seeded on Z10 (Figure 3.3 a) surfaces developed the typical elongated morphology of adhered cells, indicating cell survival. Cell spreading parameters on Table 3.1 show that cell area increased for Z10, cell perimeter increased for Z2 - Z10, and circularity decreased also for Z2 –Z10, suggesting a possible role of zein on cell spreading.

Cell viability was measured with a CKK 8 kit, which determines viability from cellular dehydrogenase activity. Cell viability (Table 3.2) increased for Z2 - Z10 surfaces with respect to Z0 in serum deprived medium, measured 9h after seeding (longer incubation time was impractical). Table 3.2 shows that cell viability also increased for Z2 - Z10, when cells were grown in serum supplemented serum (measured 20h after seeding). Results suggested that zein coatings enhanced cell viability. Dong et al. (2004) reported that lower zein concentration, 0.3%, had higher cell adhesion than 0.9%. In their work, coatings were prepared by casting rather than immersion which resulted in much higher zein content. Their report suggested there is a limit to zein content in coatings that favor cell adhesion.

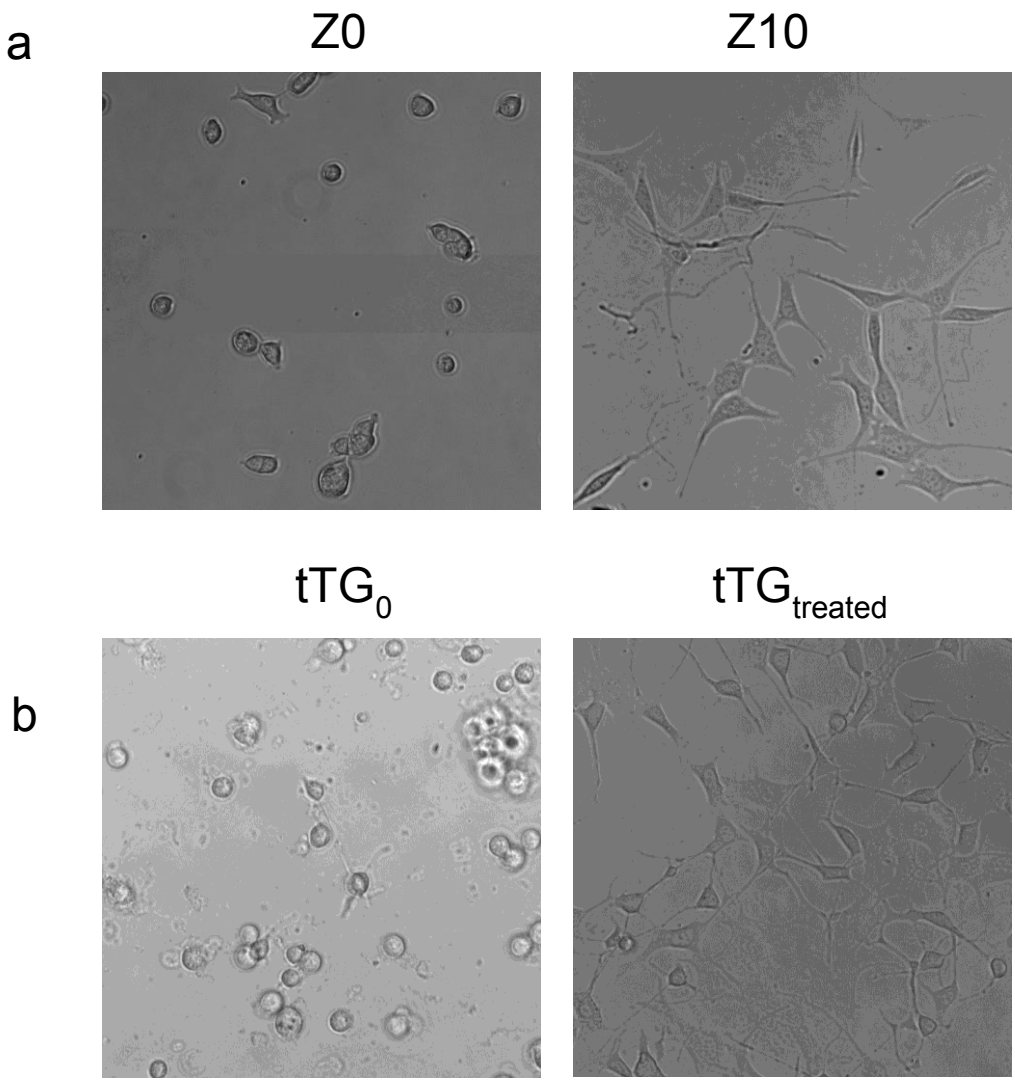

Figure 3.3. Inverted microscope image of 3T3 cells grown in serum deprived medium on (a) zein coated cover slips (images taken at 15h after cell seeding, 200X); and (b) zein coated cover slips treated with tTG (images taken at 6h after cell seeding, 200X).

3.4.3 Effect of tTG treated zein surfaces on cell spreading and viability

Cell spreading parameters on Table 3.3 show an increase in area, perimeter, and lower circularity for tTG treated zein surfaces with respect to tTG_0, in serum deprived and serum supplemented media. Serum deprivation led to cell degeneration and abnormal morphology in tTG_0 (Figure 3.3b), but 3T3 grown on tTG treated surfaces developed the typical spindle morphology indicative of cell spreading (Figure 3.3b, $tTG_{treated}$). Cell spreading parameters suggested that tTG enhanced cell spreading, especially considering its effect on serum deprived cells where no exogenous adhesion proteins were available to promote spreading. It is possible that tTG and zein had interactive roles in 3T3 cell adhesion and spreading. The viability of 3T3 (Table 3.2) was significantly higher on zein surfaces treated with tTG than on control surfaces in serum supplemented (13h incubation) and serum deprived media (6h incubation), again suggesting a role of tTG in cell spreading, adhesion and viability.

Table 3.1. Cell spreading parameters for 3T3 cells seeded on zein coated cover slips ($p<0.05$).

	Serum supplemented medium			Serum depleted medium		
Group	Area (μm^2)	Perimeter (μm)	Circularity	Area (μm^2)	Perimeter (μm)	Circularity
Z0	865.9 ± 67.5^{a}	162.9 ± 7.2^{a}	0.50 ± 0.02^{a}	272.4 ± 17.9^{a}	68.5 ± 3.4^{a}	0.8 ± 0.03^{a}
Z0.25	1268.4 ± 140.2^{b}	261.8 ± 12.6^{b}	0.20 ± 0.02^{b}	264.8 ± 16.6^{a}	77.4 ± 5.5^{a}	0.7 ± 0.04^{a}
Z0.5	1494.1 ± 169.9^{b}	298.3 ± 17.4^{bc}	0.26 ± 0.02^{b}	242.3 ± 14.4^{a}	68.8 ± 3.9^{a}	0.7 ± 0.03^{ab}
Z2	1340.0 ± 95.8^{b}	297.3 ± 14.8^{bc}	0.23 ± 0.02^{b}	271.8 ± 17.2^{a}	99.9 ± 7.2^{b}	0.5 ± 0.04^{c}
Z10	1594.8 ± 143.0^{b}	319.0 ± 16.8^{c}	0.23 ± 0.02^{b}	704.1 ± 58.1^{b}	217.1 ± 11.3^{c}	0.2 ± 0.01^{d}

Table 3.2. Effect of zein coating and tTG treatment on cell viability ($p<0.05$).

Zein coating		
Group	Serum supplemented medium	Serum depleted medium
Z0	0.453 ± 0.018^{a}	0.358 ± 0.006^{a}
Z0.25	0.440 ± 0.003^{a}	0.344 ± 0.003^{a}
Z0.5	0.444 ± 0.006^{a}	0.356 ± 0.005^{a}
Z2	0.504 ± 0.003^{b}	0.377 ± 0.008^{b}
Z10	0.507 ± 0.006^{b}	0.396 ± 0.003^{c}
tTG treatment		
Group	Serum supplemented medium	Serum depleted medium
tTG_0	0.402 ± 0.007^{a}	0.441 ± 0.028^{a}
$tTG_{treated}$	0.446 ± 0.005^{b}	0.772 ± 0.065^{b}

Table 3.3. Cell spreading parameters for 3T3 cells seeded on zein treated with tTG ($p<0.01$).

	Serum supplemented medium			Serum depleted medium		
Group	Area (μm^2)	Perimeter (μm)	Circularity	Area (μm^2)	Perimeter (μm)	Circularity
tTG_0	540.2 ± 87.2^{a}	142.7 ± 14.7^{a}	0.5 ± 0.04^{a}	263.0 ± 12.8^{a}	59.5 ± 1.5^{a}	0.9 ± 0.004^{a}
$tTG_{treated}$	1546.3 ± 201.6^{b}	270.8 ± 19.9^{b}	0.3 ± 0.03^{b}	664.9 ± 60.1^{b}	188.2 ± 20.3^{b}	0.27 ± 0.2^{b}

Table 3.4. SDS-PAGE band intensity of zein, tTG, and zein treated with tTG.

Protein bands	KDa	SDS-PAGE band net intensity (×1000)*		
		Zein	tTG	Zein-tTG
α-19	19	2825 ± 52[a]		2050±16[b]
α-22	22	3711±257[a]		2778±103[b]
tTG	75		1326±145[a]	860±18[b]
	100		432±20[a]	845±18[b]
	> 250		2583±13[a]	4640±39[b]

*****p<0.1 for comparisons within protein bands.

3.4.4 Zein and tTG interaction

The possibility of cross-linking between tTG and zein was evaluated by SDS-PAGE. Zein, in this study, consists mainly of α-zein, including α-19 (19kDa) and α-22 zein (22kDa). Zein tends to form oligomers 38-48kDa and above 88kDa (Nonthanum, 2012). The molecular weight of tTG is 76.6 kDa (Ikura, 1988).

Quantification of gel bands is presented on Table 3.4. Intensity values are averages of replicate wells. Zein was treated with tTG (12.9 µg/well). Zein shows typical bands 19 and 22kDa. tTG band showed at 75kDa. When zein and tTG were combined, the intensity of α-19, α-22 and tTG bands decreased, while intensity increased for 100kDa and >250kDa bands. The increased intensity of 100kDa band was interpreted as representing a 1:1 cross-linking between zein and tTG. The large molecular weight band at >250kDa may represent complex polymerization between zein and tTG units. It is likely that zein may have multiple glutamine reactive sites for tTG. An image of the SDS-PAGE gel is provided on Appendix Figure B.3.

3.4.5 Incorporation of dansylcadaverine (MDC) into zein by tTG

MDC, a fluorescent compound that acts as lysine donor (Parameswaran, Velasco, Wilson, & Lorand, 1990) was utilized to further investigate if zein was a potential glutamine donor for tTG. Figure 3.4 shows the fluorescence of MDC. Zein incubated with MDC in the absence of tTG

showed no fluorescence. However, when tTG was combined with zein and MDC, zein bands appeared under UV light. Zein fluorescence was attributed to tTG mediated incorporation of MDC into zein, suggesting that zein is a glutamine donor for tTG.

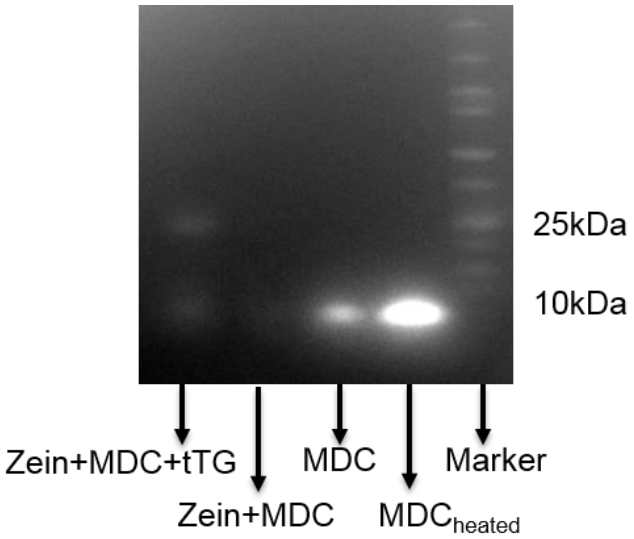

Figure 3.4. SDS-PAGE image showing the interaction between zein and MDC mediated by tTG. From left to right: zein and MDC with tTG added, zein and MDC, MDC, MDCheated, marker.

SDS-PAGE indicated cross-linking between zein and tTG, suggesting cross-linking may be involved on enhanced cell spreading and viability on zein coated surfaces. Akimov et al. (2000) explained that in the cell adhesion process, the activity of integrin was facilitated by tissue transglutaminase. By cross-linking various ECM proteins, such as fibronectin, tTG acts as a bridge between integrin and matrix proteins or may promote the formation of large complexes with the three proteins linked to each other. A possible explanation for enhanced cell spreading and viability of 3T3 cells on zein surfaces is that cross-linking between zein and tTG may have anchored tTG onto the substrate surface, thus providing a high affinity binding site for cell adhesion proteins, possibly fibronectin, an adhesive glycoprotein secreted by many types of cells, including fibroblasts (Grinnell & Feld, 1979).

Alternatively, enhanced cell adhesion by zein coatings may be related to direct interaction between α-22 zein and integrin. Some subfamilies of α-22 zein contain the cell adhesion sequence LDV, 188 LVVANPTAYLQQLLPFNQ**LDV**ANSAAYLQQRQQLLNPLAAANPLVAAFLQQQQFL

PYNQISLMNLALSRQQPIVG 262 (From NCBI, GenBank: CAA43399.1), an integrin binding motif that is found in ECM proteins such as fibronectin (Campbell & Humphries, 2011). Results of this work suggested that zein is also a substrate for tTG and their interaction promoted cell adhesion and spreading.

3.5 Conclusions

Zein, coated on oxygen plasma treated glass, enhanced cell spreading and viability of 3T3 cells with respect to uncoated glass surfaces. The mechanism for cell spreading on zein surfaces was hypothesized to involve transglutaminase attachment to the glutamine rich surface of zein. In this work, SDS-PAGE experiments indicated that zein can crosslink with tTG. Furthermore, tTG allowed zein to bind dansylcadaverine, suggested that zein is a glutamine donor for tTG. Thus, it is possible that the mechanism for zein enhanced cell spreading may be cross-linking between zein and tissue transglutaminase excreted from 3T3 cells. A combination of tTG and zein may improve the application potential of zein as a biomaterial.

Chapter IV. CTC cell capture on zein surfaces deposited with tissue transglutaminase

4.1 Abstract

Detection and characterization of circulating tumor cells (CTCs) are important for cancer diagnosis and therapy. Various methods have been proposed for CTCs capture and enumeration. We previously found that zein was the glutamine donor for tissue transglutaminase (tTG) and zein coatings on cover slip enhanced cell spreading and adhesion of NIH 3T3 cells. In the present study, MDA-MB-231 cells were used as CTC cells surrogate. Cell capture on zein and tTG deposited surface was studied. It was found that zein and tTG promoted the initial adhesion of MDA MB 231 cells. The number of capture cells increased with incubation time. Also, after 1h incubation time, the number of captured cells increased with zein content in the coating. Cell capture yield increased linearly with tTG concentration. This study provided preliminary supporting evidence for developing a zein and tTG platform for CTCs capture.

4.2 Introduction

In the process of cancer metastasis, a small amount of circulating tumor cells (CTCs) are enough to seed the metastasis colonies (Cristofanilli, 2006). These cells are responsible for cancer propagation and thus represent a major challenge to cancer treatment (Cristofanilli, 2006). CTCs are used as “liquid biopsy”. Various studies have reached the conclusion that stem cell markers are present in CTCs. Enumeration of CTCs provides prognostic information for early stage cancer patients (Balic et al., 2012; Wicha et al., 2006). Molecular analysis of CTCs may assist in treatment strategy (Liling Zhang et al., 2012).

In order to enhance clinical diagnosis, isolation CTCs from patients is important. Blood is a cost-effective source of CTCs. One of the biggest challenges for CTCs detection is that the CTCs percentage among all blood components is very low. It is estimated that there is one CTC in 10^7 white blood cells per milliliter of blood (Krebs et al., 2014). Thus, various techniques aimed at enriching and detecting CTCs have been developed (Krebs et al., 2014). They can be divided into two groups: immunoaffinity isolation and physical methods. For immunoaffinity based isolation, an epithelial cell adhesion molecule (EpCAM) is exploited for separating epithelial CTCs from the blood cells because it is expressed in epithelial cells, but not in blood cells (Balic et al., 2012;

Balzar, Winter, de Boer, & Litvinov, 1999; Nagrath et al., 2007b; Went et al., 2004). However, this system is not sensitive for detecting cells that undergo epithelial to mesenchymal transition (EMT), thus assays based on this marker underestimated CTCs numbers in late metastatic patients (Nagrath et al., 2007). Besides, CTCs demonstrated heterogeneous phenotype. For example, CTCs from breast cancer patients do not have EpCAM expression (Gorges et al., 2012; Krebs et al., 2014). Physiochemical methods utilize differences in physiochemical properties such as size, density and deformability between CTCs cell and blood cells (Yu et al., 2013; Yu, Stott, Toner, Maheswaran, & Haber, 2011; Lixin Zhang et al., 2013) However, these methods also have limitations. For example, CTCs are generally assumed to be larger than nucleated blood cells, but CTCs at apoptosis or dormant stage have smaller sizes (Harouaka, Nisic, & Zheng, 2013). Also, both, immunoaffinity isolation and physiochemical methods are expensive methods.

Nanostructured materials have been recently explored as a platform for CTC capture. They provide more surface area, thus increase cell-substrate interactions and enhance cell capture efficiency (Fischer et al., 2009; S. Wang et al., 2011). As a natural abundant biomaterial, zein has been used to generate nanostructured surfaces in food and pharmaceutical applications. We previously found that zein was the glutamine donor of tTG, a widely distributed enzyme expressed by various cell types including MDA MB 231. tTG mediate crosslinking between glutamine residues on one polypeptide and the lysine residues of another polypeptide (Laszlo Lorand & Graham, 2003b). It enhances cell adhesion and spreading (Belkin et al., 2005a; Chau et al., 2005b; Jones et al., 1997). The objective of this work is to investigate if zein substrates, maybe aided by tTG, can be effective in capturing cancer cells.

4.3 Materials and methods

4.3.1 Surface coating

Glass cover slips (VWR, Rednor, PA), 5 or 18 mm in diameter, were rinsed with isopropanol and placed in 12 or 96 well micro plates. Plates were exposed to oxygen plasma treatment (Diener Electronic PICO, Lomazzo, Italy) at power level of 100W and chamber pressure of 0.8-1 mbar, for 1 min. Coatings were prepared by dissolving 0.5mg/ml (Z0.5) and 10mg/ml (Z10) zein (Showa, Sangyo, Japan) in 75% isopropanol with stirring (on stir plate for 30min), pH adjusted to 4.0±0.5 with acetic acid (ACROS, New Jersey), and then pipetted into micro plates (2ml for 12 well plate and 200ul for 96 well plate). After 1h incubation at room temperature, the solution was discarded.

Wells were rinsed with Milli Q water and dried. Oxygen plasma treated cover slips without zein coating served as control surfaces (Z0).

For the tTG treatment group, tTG solution was prepared fresh by dissolving 0.15mg/ml tissue transglutaminase (Sigma, St Louis, MO) in tTG buffer containing 1mM Dithiothreitol (Sigma, St Louis, MO), 3mM $CaCl_2$ (Fisher Scientific, Waltham, MA) and 10mM Tris-HCl pH 8.3. Zein coated coverslips (5mm diameter) prepared as above (Z0.5) were further incubated in tTG solution for 2h at 37˚C in a 5% CO_2 incubator. Wells were washed, dried and UV sterilized for 15min before cell seeding. A zein coated cover slip treated with tTG buffer was used as control (tTG_0).

4.3.2 Cell culture

MDA-MB-231 cell line was cultured in L-15 medium (Fisher-Mediatech, Manassas, VA) supplemented with 10% bovine calf serum (Life Tech, Carlsbad, CA) and 1% penicillin/streptomycin (Fisher-Mediatech, Manassas, VA) at 37˚C in a humidified incubator. Cell line was used in low passage number 2-4.

4.3.3 Cell spreading assay

MDA-MB-231 cells were trypsinized, collected in serum supplemented or serum deprived medium and seeded into 96 or 12 well plate at a density of 10^4 or 2×10^4 cells/ml (100ul or 2ml). Cell morphology images were taken with an inverted microscope (Olympus IX81, Tokyo, Japan). Cell outlines were manually traced by Image J software (National Institute of Health, Bethesda, MD). Cell morphology parameters including area, perimeter and circularity were automatically measured after tracing. Circularity was calculated as 4π (area/perimeter^2). A circularity value of 1 means a perfect circle. For tTG treated substrates, cells were seeded in serum supplemented or serum deprived medium at a density of 10^4 or 10^5 cells/ml (100ul, 96 well plate).

4.3.4 Cell adhesion assay

At confluency, cells were trypsinized and collected in serum supplemented or serum deprived medium and seeded into 96 well plate at density of 10^4 or 10^5 cells/ml (100ul). The viability of attached cells was quantitatively determined with Cell Counting Kit 8 (Dojindo, Rockwille, MD), according to the manufacturer's instructions. The medium was discarded after incubation and wells were washed with 1×PBS before fresh medium was pipetted in. Cell Counting Kit 8 reagent (10ul)

was added into each well and incubated for 2h at 37˚C. Absorbance was measured with a micro plate reader (BioTek Instruments, Winooski, VT) at 450nm.

4.3.5 Cell capture yield

To determine capture yield, wells were gently washed with PBS to remove the loosely attached cells. Then, cells were imaged with an inverted microscope. Images were randomly taken from each replicate well. Cell number in each well was counted and cell density was calculated as number of cells per mm^2. The final capture yield was defined as the captured cell number/initial cell seeded number.

4.3.6 Statistical analysis

Differences between datasets were analyzed with Student's *t-test* and Two-way ANOVA (SAS institute, Cary, NC).

4.4 Results and discussion

4.4.1 Effect of zein coating on cell spreading

Cell spreading parameters, including area, perimeter and circularity were measured to explore whether zein coated cover slips can capture CTCs. MDA MB 231, a metastatic breast cancer cell line isolated from pleural effusions of a breast cancer patient, was used in this study (Cailleau et al., 1974). According to Figure 4.1.a, MDA-MB-231 cells grown on oxygen plasma treated glass substrate under serum deprivation, were mostly round. After the surface was treated with zein, more cells developed a spindle shape. Quantification of cell spreading parameters from images in Figure 4.1.b also showed that cell area and perimeter increased significantly on zein coated surfaces, while circularity decreased significantly. Cell adhesion was measured, but was not found significant between Z0 and Z10.

In Chapter III, it was concluded that zein was a glutamine donor for tTG. The enhanced cell spreading on zein surface may be associated with tTG excreted from MDA MB 231. It is likely that tTG mediated cell spreading through the interaction with zein deposited on the substrate.

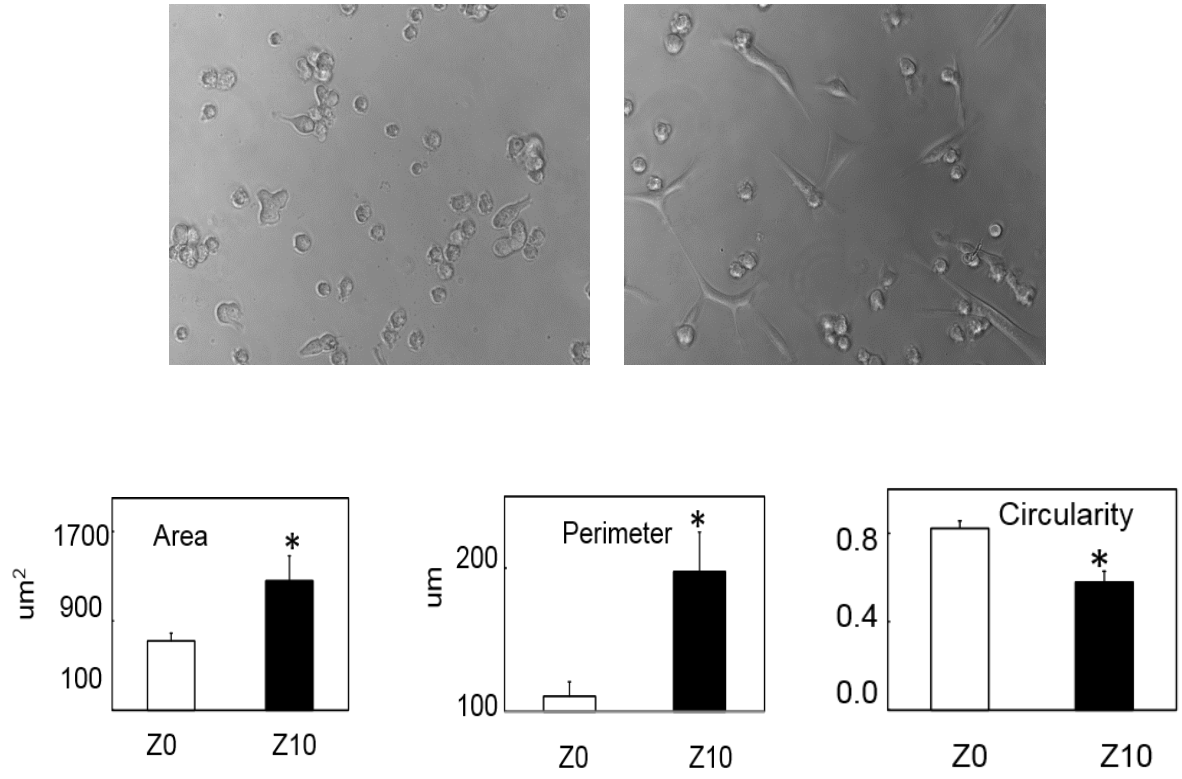

Figure 4.1. Inverted microscope image of MDA-MB-231 cells grown on zein coated cover slips in serum deprived medium (images taken at 36h, 200X). (b) Quantification of cell spreading parameters (*$p<0.05$).

4.4.2 Effect of tTG coating on cell spreading and adhesion

To further confirm the effect of tTG on cell spreading and adhesion, tTG was deposited on zein coated cover slip (Z0.5). Figure 4.2 shows that tTG coatings markedly enhanced cell spreading for cell cultured in both serum supplemented and serum deprived media. Cells seeded on zein without TG showed a round morphology, indicative of poor attachment. However, when TG was present, MDA-MB-231 cells adopted a flat morphology, typical of adhered MDA-MB-231 cells. For serum deprived cells, the effect of TG was less apparent, although still significant. The quantification of cell spreading parameters also shown in Figure 4.2 a, b, indicated that tTG coating increased cell area and perimeter and decreased circularity. Figure 4.3 shows that TG coatings increased cell attachment. It was proposed that tTG improved cell adhesion by crosslinking zein with cell matrix protein proteins.

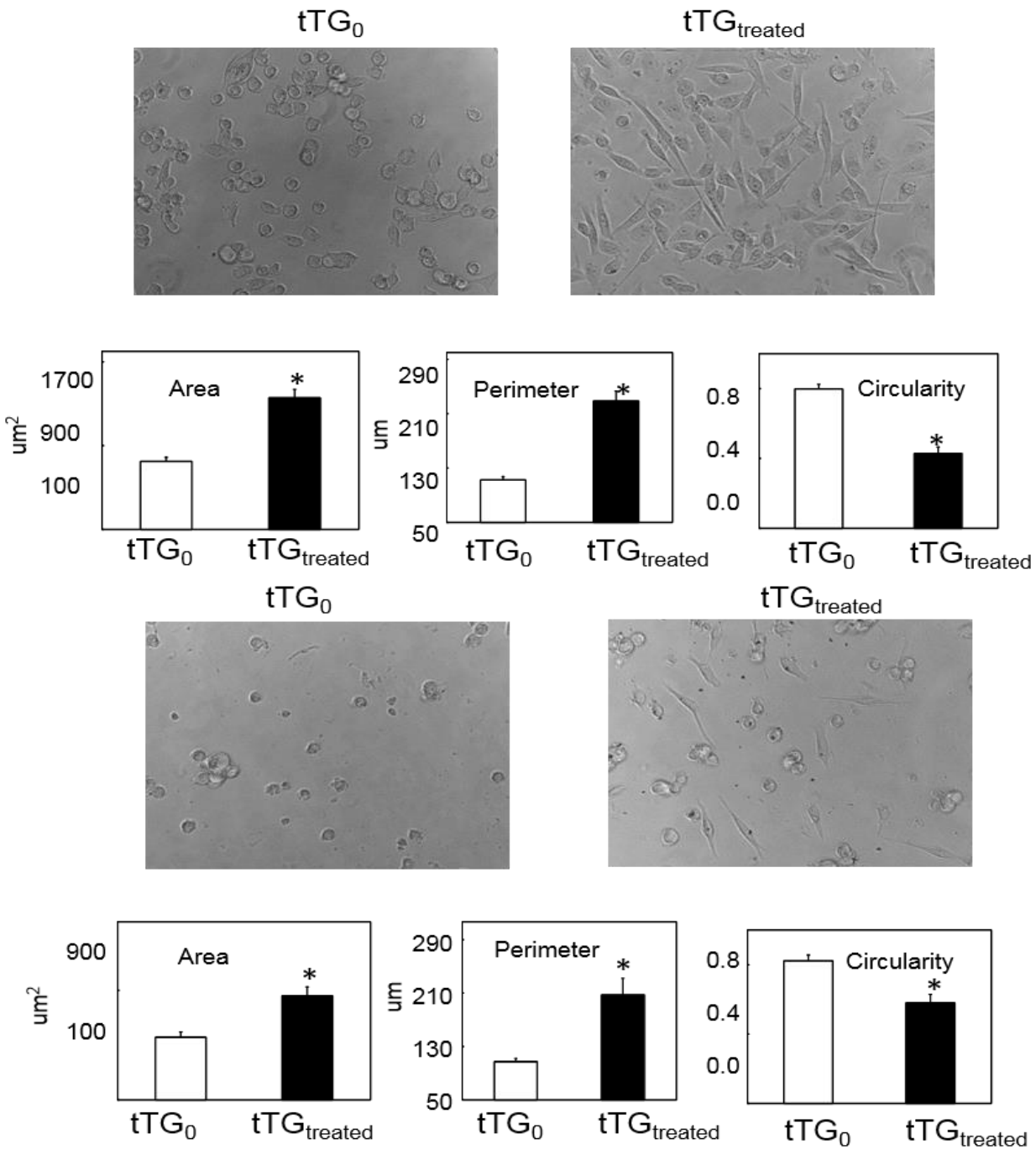

Figure 4.2. (a) Inverted microscope images of MDA-MB-231 cells grown on zein coated cover slips treated with tTG, in serum containing medium (images taken at 18h, 200X). (b) Quantification of cell spreading parameters. (c) Inverted microscope images of MDA-MB-231 cells grown on zein coated cover slips treated with tTG, in serum deprived medium (images taken at 40h, 200X). (d) Quantification of cell spreading parameters (*$p<0.05$).

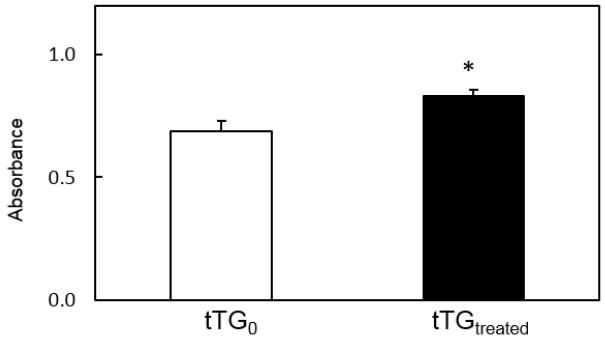

Figure 4.3 Effect of tTG on MDA-MB-231 cell adhesion. MDA-MB-231 cells grown on zein coated cover slips treated with tTG, in serum containing medium for 19h (*p<0.05).

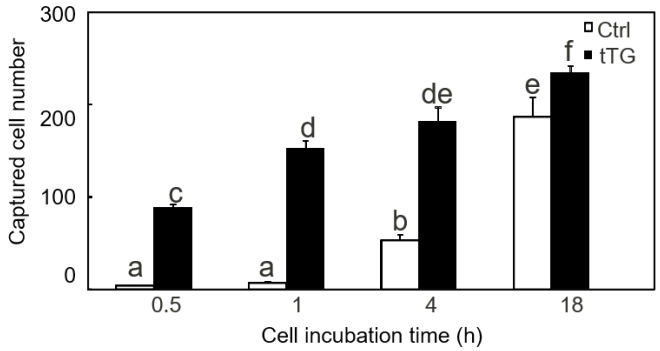

Figure 4.4 Effect of incubation time on captured cells. MDA-MB-231 cells were seeded on tTG treated Z0.5 (*p<0.05).

4.4.3 Effect of incubation time on cell capture

Figure 4.4 shows the effect of incubation time, 0.5h, 1h, 4h and 18h, on the number of cells captured with and without tTG on the substrate. At 0.5h, cell capture for the substrate with tTG was nearly 20 times higher than for the substrate without it. In the tTG group, cell capture increased significantly from 0.5h to 1h and also from 4h to 18h. In the group with no tTG on the substrate, cell capture increased significantly from 1h to 4h and from 4h to 18h. tTG treated substrates captured significantly higher number of cells within 18h after incubation compared with substrates with no tTG. Meanwhile, the difference between the two groups decreased with time. This may due to increasing cell excretion of tTG into the extracellular matrix. Thus, zein substrates coated with tTG improved cell adhesion in the short run.

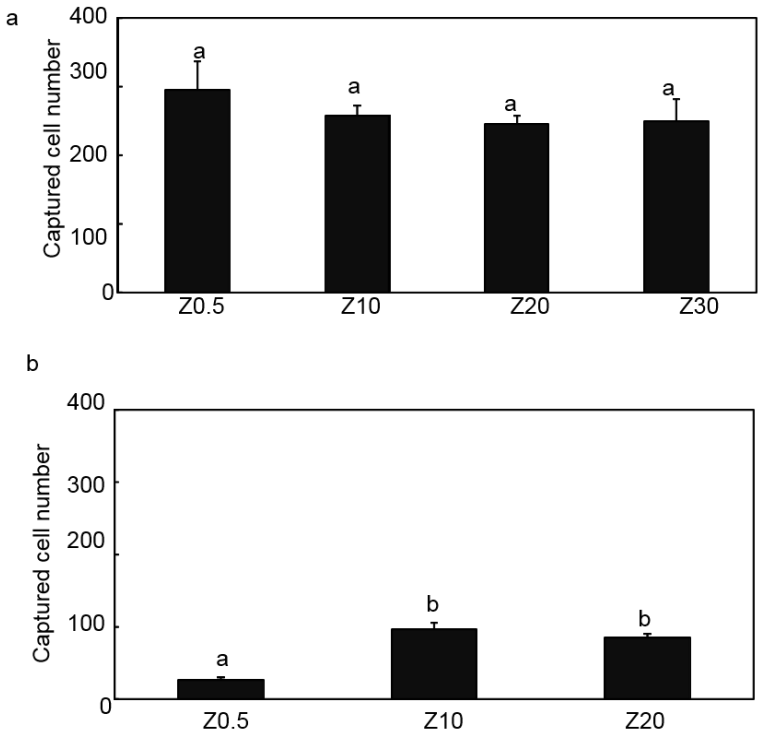

Figure 4.5 Effect of zein content on captured cells. MDA-MB-231 cells were seeded on tTG treated Z0.5 (*p<0.05). (a) 18h cell incubation, (b) 1h cell incubation

4.4.4 Effect of zein content on cell capture

In order to determine the effect of zein content of substrate on cell capture, Z0.5, Z10 and Z20 substrates were treated with tTG. Cell capture was observed after 1 h and after 18h. Figure 4.5 shows nearly a 4 fold increase in cell capture from Z0 to Z10 after 1 h of incubation. However, no further increase in cell capture was observed for Z20. It is possible that the Z0.05 substrate was sparsely coated and the amount of accessible zein was not enough to interact with tTG. Zein content on Z10 may have allowed better utilization of tTG increasing cell capture over Z0.5 This result is consistent with the hypothesis that zein and tTG play complementary roles in cell adhesion. On the other hand, no differences in cell capture were observed among Z0.5, Z10, and Z20 after 18h of incubation. This suggested that zein was involved in the initial adhesion of MDA MB 231. By 18h, cells may have excreted fibronectin or tTG and thus the presence of zein on the substrate was no longer critical.

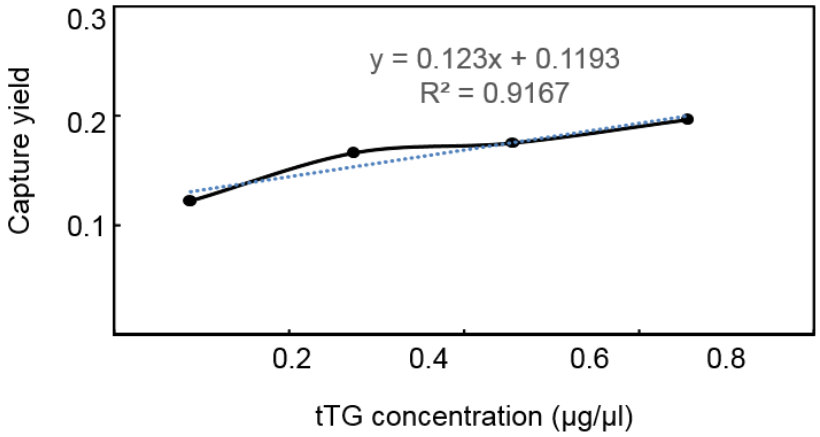

Figure 4.6 Effect of tTG concentration on cell captured yield. MDA-MB-231 cells were seeded on tTG treated on Z5 cover slip (17h after cell incubation).

4.4.5 Effect of tTG concentration on cell capture

To study the effect of tTG concentration on cell capture, Z0.5 substrates were treated with tTG solutions at 0.088mg/ml, 0.275mg/ml, 0.456mg/ml and 0.656 mg/ml. Around 20 images were taken from each group at after 17h of incubation. Cell capture yield was calculated based on the average number of cells obtained. Cell capture yield on the four surfaces were respectively 12.2%, 16.6%, 17.5% and 19.6%. Figure 4.6 shows that capture yield increased linearly with tTG concentration for the range studied.

4.5 Conclusions

In this study, zein observed to enhance MDA MB 231 cell spreading. tTG treatment of zein substrates further increased cell spreading. The affinity of MDA-MB-231 with zein films may confer them cancer cell retrieval properties.

Although many CTCs capture devices have been devised, there is still an urgent need to develop an inexpensive, efficient, and convenient method. In order to test whether zein films are promising substrates for CTC isolation, it is necessary to test the affinity of blood cells (red blood cells, white cells, and platelets) to zein films and tTG treated zein films. If the use of microfluidic devices is contemplated, it is also important to test blood cells affinity to zein films under shear stress.

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Appendix A. Inverted microscope images of 3T3 cells

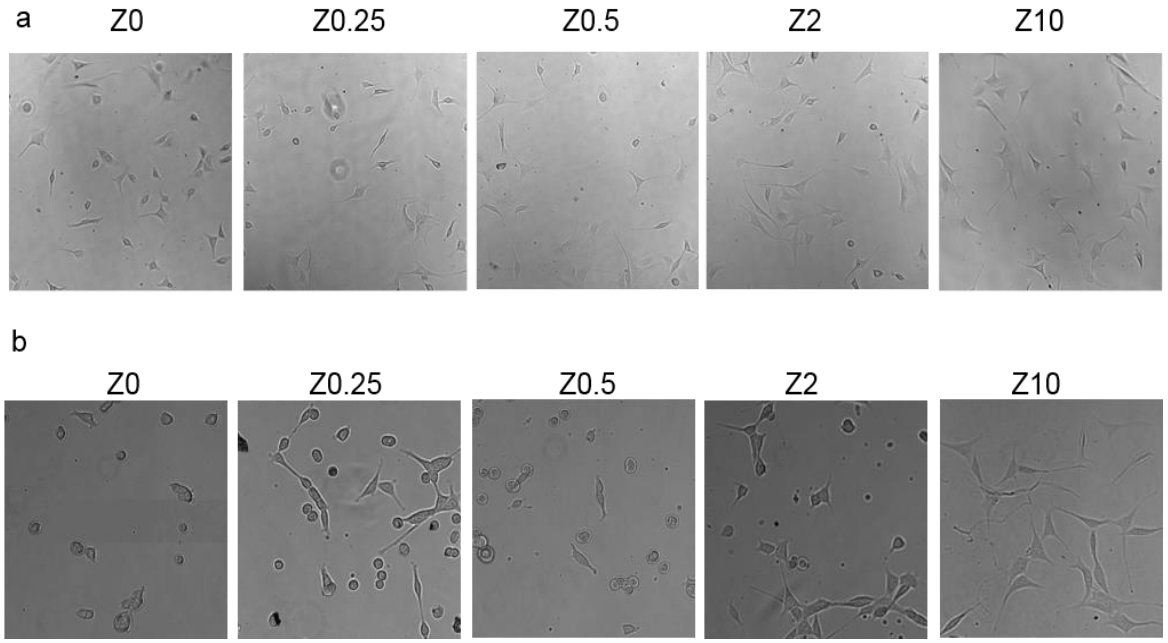

Figure A.1 Inverted microscope image of 3T3 cells grown in (a) serum supplemented medium (100X); (b) serum deprived medium (200X) on zein coated cover slips. Images were taken 15h after cell seeding.

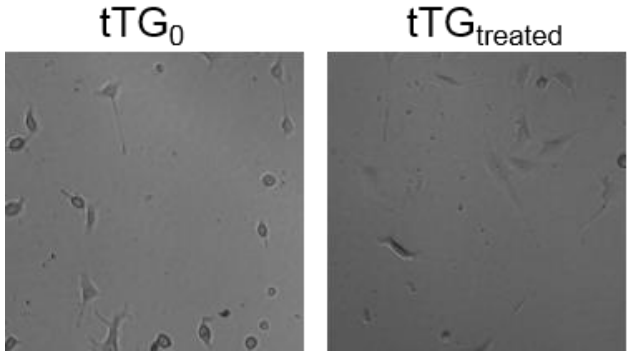

Figure A.2 Inverted microscope image of 3T3 cells grown in serum supplemented medium on zein coated cover slips treated with tTG. (100X).. Images were taken 13 h after cell seeding.

Appendix B. Data on zein solubility, cell adhesion, substrate characterization and zein-tTG interaction

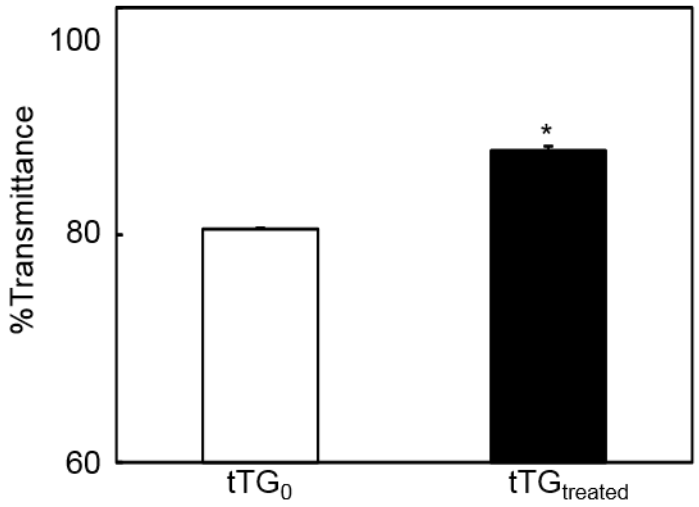

Figure B.1 Transmittance of zein solution in the absence of tTG (tTG0) and presence of tTG (tTGtreated).

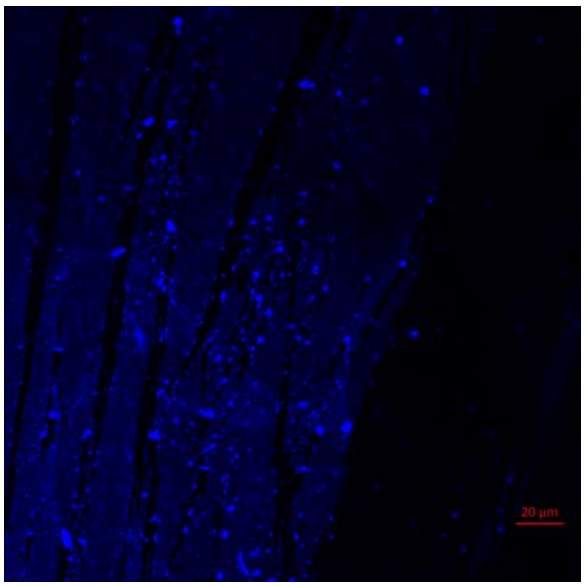

Figure B.2 Confocal image of zein coated cover slip, Z10.

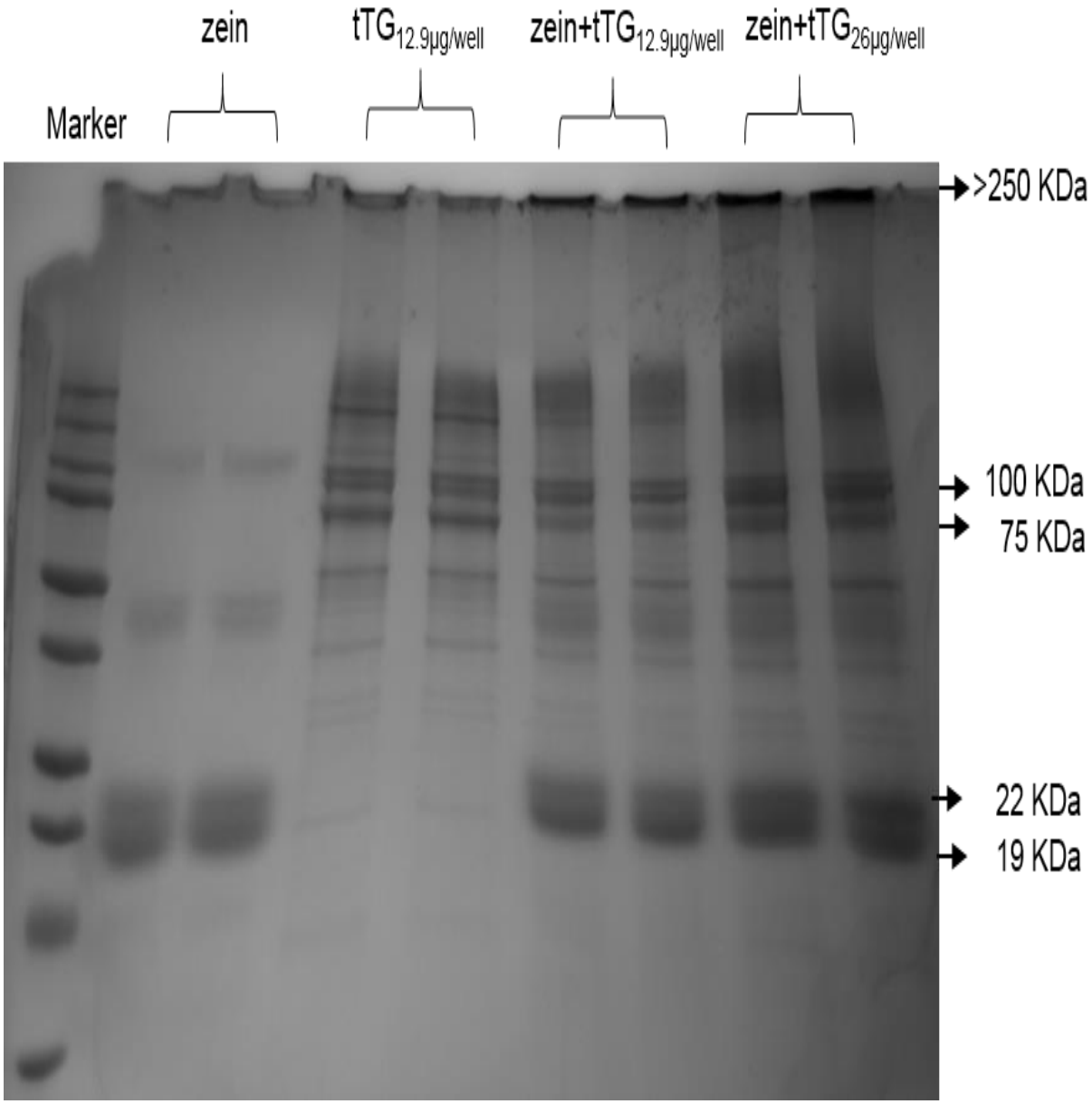

Figure B.3 SDS-PAGE image of zein treated with tTG. From left to right: marker (lane 1), zein (lanes 2, 3), $tTG_{12.9\mu g/well}$ (lanes 4, 5), zein treated with $tTG_{12.9\mu g/well}$ (lanes 6, 7), zein treated with $tTG_{26\mu g/well}$ (lanes 8, 9).

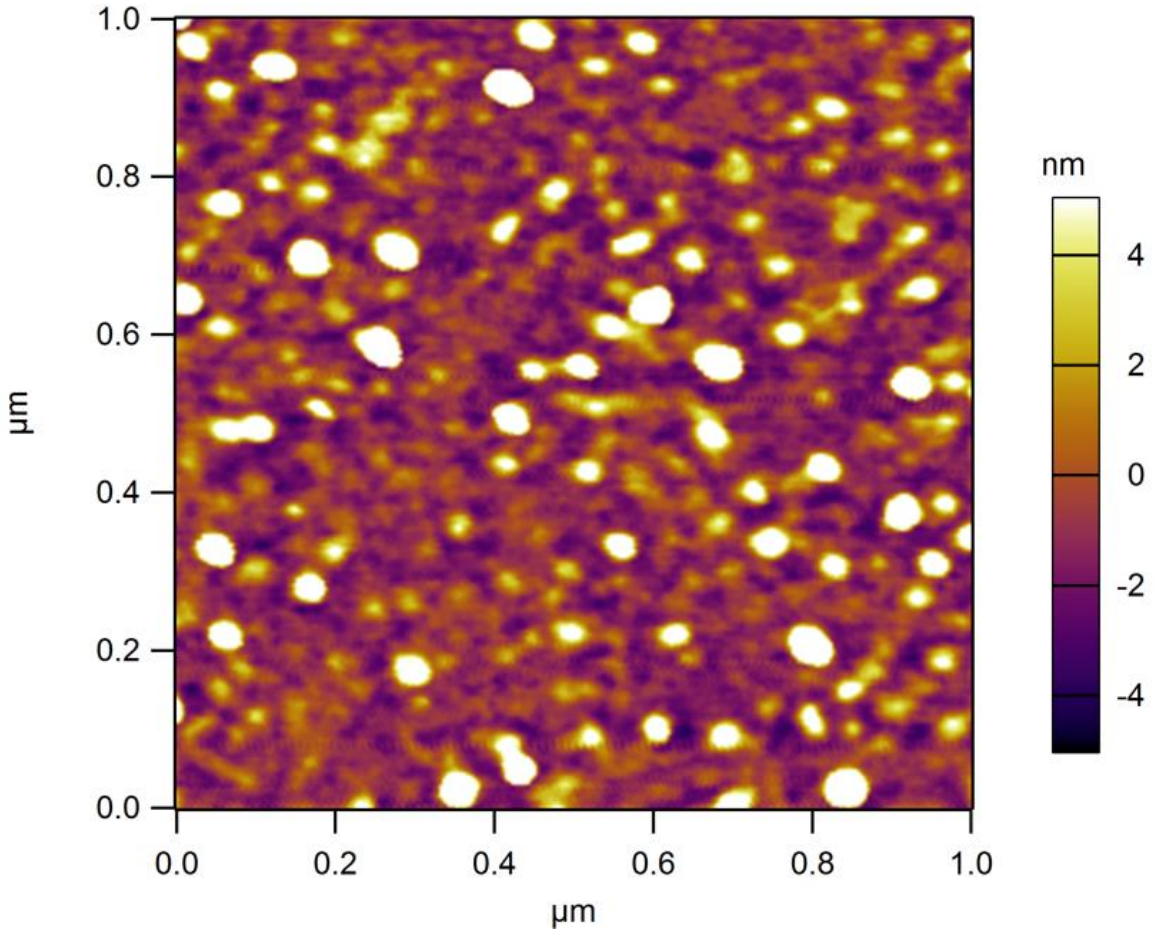

Figure B.4 AFM image of Z0.5 substrate

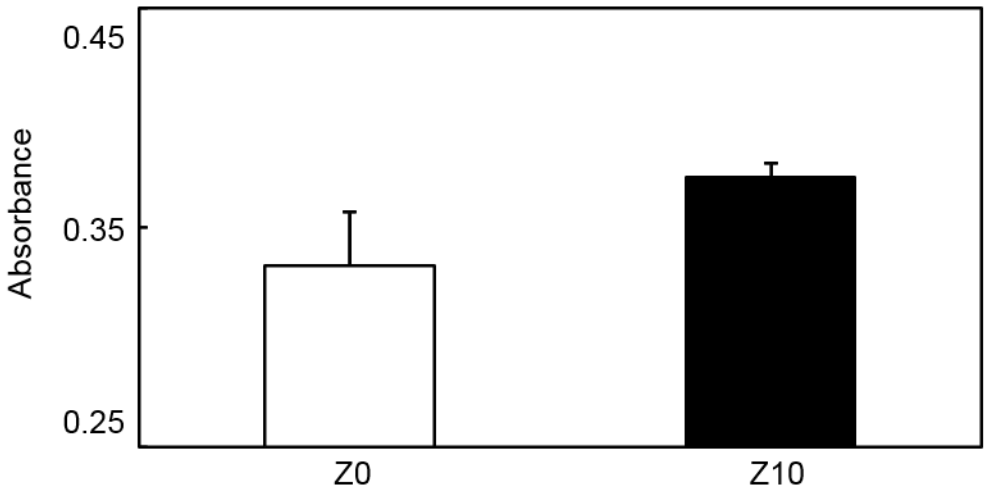

Figure B.5 Effect of zein on MDA-MB-231 cell adhesion. MDA-MB-231 cells grown on zein coated cover slips in serum deprived medium.

Appendix C. Approval of copyright materials

License Number	3897111497226
License date	Jun 27, 2016
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Licensed Content Title	Cell spreading and viability on zein films may be facilitated by transglutaminase
Licensed Content Author	Hemiao Cui, Gang L. Liu, Graciela W. Padua
Licensed Content Date	1 September 2016
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Licensed Content Issue Number	n/a
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