1 **Cell spreading and viability on zein films**
2 **may be facilitated by transglutaminase.**
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31 **Abstract**

32 Zein is a biocompatible corn protein potentially useful in the development of biomaterials. In this
33 study, the deposition of zein on oxygen plasma treated glass cover slips significantly enhanced
34 cell spreading and viability. The mechanism for cellular response to zein coated surfaces was
35 thought to involve the polyglutamine peptides on the zein structure. We hypothesized that zein
36 was a substrate for tissue transglutaminase (tTG), an extracellular enzyme involved in cell-
37 surface interactions. SDS-PAGE results suggested an interaction between zein and tTG, where
38 zein was the glutamine donor. Cross-linking between zein and tTG may be the first step in
39 successful cell adhesion and spreading.
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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 [1–3]. Deletion of these proteins is often associated with loss of cell adhesion [2,4]. Bioactive proteins adhere to surfaces based on their physicochemical properties and their adsorption patterns impact cellular response [1]. 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 [1]. When cells adhere to surfaces but show no spreading, cell apoptosis is increased [5]. 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 [6]. The enzyme is expressed by various cell types including NIH 3T3 and excreted to the cell surface and extracellular matrix (ECM) [7–9]. 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 [10]. Ample evidence indicates that tTG mediated cross-linking plays a crucial role in enhancing cell adhesion and spreading [11–13]. 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 [14]. Examples of tTG substrates include fibronectin and collagen [15,16]. 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 [17–19]. Also, the continuous glutamine stretches need to be accessible and away from discouraging residues [20].

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 [21]. 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 [21,22]. Specifically, zein has been applied in food and medical fields in the forms of film, fibers and nanoparticles [21]. Zein films demonstrated better biocompatibility than Corning plates with human umbilical veins endothelial cells [23]. Plasmid DNA encapsulated in zein spheres was protected from degradation by DNase I and was steadily released over more than 7 days [24]. Additionally, hollow zein spheres fabricated from phase separation were applied to increase drug loading capacity [25].

Interestingly, glutamine is an abundant amino acid at the zein surface, forming polyglutamine strands [26,27]. 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 [27]. The tandem repeating units form α -helices arranged in anti-parallel stacks. The neighboring helices are connected with glutamine turns [27,28]. Figure 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 [28]. 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 [29], it is not unreasonable to investigate whether zein could be a possible substrate of tTG.

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.

Materials and methods

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 [30], 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 CaCl₂ (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% CO₂ 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 (tTG₀).

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%, CO₂ incubator. Cell line was used in low passage number.

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 10⁴ or 2×10⁴ 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 $4\pi \text{ (area/perimeter}^2\text{)}$. 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).

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, Rockville, 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% CO₂. Absorbance was measured with a micro plate reader (BioTek Instruments, Winooski, VT) at 450nm.

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% CO₂, 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).

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% CO₂ incubator for 2h. After dilution with 2×Laemmli sample buffer, proteins were separated by SDS-PAGE. Gel image was taken under both UV light and white light and then merged.

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).

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.

Statistical Analysis

Differences between datasets were analyzed using Student's *t*-test and Two-way ANOVA (SAS institute, Cary, NC). Significance level was set at $p < 0.05$.

Results and discussion

Surface Characterization

AFM image of zein coated glass cover slip (Z2) showed a uniform topography (Figure 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 2b) shows the formation of scattered zein spheres on top of the films. Film formation was confirmed with Nile blue staining (see Supplementary Figure 1). Wang et al [31] showed that zein adsorption to hydrophilic self-assembled monolayers formed by 11-mercaptopundecanoic 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.

Effect of zein coatings on cell spreading and viability

Wang et al. [30] reported adhesion and spreading of 3T3 cells on zein hydrophilic films as opposed to zein on hydrophobic surfaces. 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 1) making an oriented layer of zein molecules with the opposite glutamine rich surface accessible to adhering cells.

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[32]. Elter et al [33] 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 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 1. Kulkarni and McCulloch [4] 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 3a). However, serum deprived 3T3 cells seeded on Z10 (Figure 3a) surfaces developed the typical elongated morphology of adhered cells, indicating cell survival. Cell spreading parameters on Table 1 show that cell area increased for Z10, cell perimeter increased for Z2 - Z10, and circularity decreased also for Z2 –Z10, suggesting that above a threshold, zein enhanced cell spreading.

Cell viability was measured with a CKK 8 kit, which determines viability from cellular dehydrogenase activity. Cell viability (Table 2) increased for Z2 - Z10 surfaces with respect to Z0 in serum deprived medium, measured 9h after seeding (longer incubation time was impractical). Table 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 [34] 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. Dong's report [34] suggested there is a limit to zein content in coatings that favor cell adhesion.

Effect of tTG treated zein surfaces on cell spreading and viability

Cell spreading parameters on Table 3 show an increase in area, perimeter, and lower circularity for tTG treated zein surfaces with respect to tTG₀, in serum deprived and serum supplemented media. Serum deprivation led to cell degeneration and abnormal morphology in tTG₀ (Figure 3b), but 3T3 grown on tTG treated surfaces developed the typical spindle morphology indicative of cell spreading (Figure 3b, tTG_{treated}). Cell spreading parameters suggested that tTG enhanced 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 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.

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 [35]. The molecular weight of tTG is 76.6 kDa [36].

Quantification of gel bands is presented on Table 4. Intensity values are averages of replicate wells. Zein was treated with tTG (12.9 μ g/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 Supplementary Figure 2.

Incorporation of dansylcadaverine (MDC) into zein by tTG

MDC, a fluorescent compound that acts as lysine donor [37] was utilized to further investigate if zein was a potential glutamine donor for tTG. Figure 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.

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 [14] 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 [38].

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
LVVANPTAYLQQLLPFNQLDVANSAAYLQQRQQLLNPLAAANPLVAAFLQQQQFL
PYNQISLMNLALSRQQPIVG 262 (From NCBI, GenBank: CAA43399.1), an integrin binding motif that is found in ECM proteins such as fibronectin [39]. Results of this work suggested that zein is also a substrate for tTG and their interaction promoted cell adhesion and spreading.

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.

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Figure Captions

Figure 1. α -Zein structural model (adapted from Matsushima et al, 1997).

Figure 2. (a) AFM image of zein coating Z2 and (b) SEM image of zein coating Z10.

Figure 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).

Figure 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, MDC_{heated}, marker.

Figure1

Figure 2

Figure 3

Figure 4

Table 1. Cell spreading parameters for 3T3 cells seeded on zein coated cover slips.

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

Table 2. Effect of zein coating and tTG treatment on cell viability.

Zein coating		
Group	Serum supplemented medium	Serum depleted medium
Z0	0.453 $\pm$ 0.018 ^a	0.358 $\pm$ 0.006 ^a
Z0.25	0.440 $\pm$ 0.003 ^a	0.344 $\pm$ 0.003 ^a
Z0.5	0.444 $\pm$ 0.006 ^a	0.356 $\pm$ 0.005 ^a
Z2	0.504 $\pm$ 0.003 ^b	0.377 $\pm$ 0.008 ^b
Z10	0.507 $\pm$ 0.006 ^b	0.396 $\pm$ 0.003 ^c
tTG treatment		
Group	Serum supplemented medium	Serum depleted medium
tTG ₀	0.402 $\pm$ 0.007 ^a	0.441 $\pm$ 0.028 ^a
tTG _{treated}	0.446 $\pm$ 0.005 ^b	0.772 $\pm$ 0.065 ^b

Table 3. Cell spreading parameters for 3T3 cells seeded on zein coated cover slips treated with tTG.

Group	Serum supplemented medium			Serum depleted medium		
	Area (μm^2)	Perimeter (μm)	Circularity	Area (μm^2)	Perimeter (μm)	Circularity
tTG ₀	540.2 $\pm$ 87.2 ^a	142.7 $\pm$ 14.7 ^a	0.5 $\pm$ 0.04 ^a	263.0 $\pm$ 12.8 ^a	59.5 $\pm$ 1.5 ^a	0.9 $\pm$ 0.004 ^a
tTG _{treated}	1546.3 $\pm$ 201.6 ^b	270.8 $\pm$ 19.9 ^b	0.3 $\pm$ 0.03 ^b	664.9 $\pm$ 60.1 ^b	188.2 $\pm$ 20.3 ^b	0.27 $\pm$ 0.2 ^b

Table 4. SDS-PAGE band intensity of zein, tTG, and zein treated with tTG.

Protein bands	KDa	SDS-PAGE band net intensity ($\times 1000$)		
		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