Cell Growth on Zein Surfaces Self-Assembled on Patterned Templates

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Introduction

Zein is the alcohol soluble protein of corn. Its secondary structure was investigated by circular dichroism[1] and by X-ray scattering measurements[2]. Both studies considered that the spatial-structure model for zein contains nine successive helical segments folded upon each other in an anti-parallel fashion, linked at each end by glutamine-rich turns or loops and stabilized by hydrogen bonds. The glutamine-rich turns are hydrophilic and locate on the top and bottom surfaces while the hydrophobic domains locate on the surfaces parallel to helices (see **Figure 1**). Zein tends to maintain its structure and only denatures at extreme conditions.[3] This unique structure can explain zein self-assembly properties. In previous work, zein was adsorbed onto gold surfaces modified by monolayers of 1-octanethiol and 11-mercaptoundecanoic acid, which generated $-CH_3$ and –COOH surfaces, respectively.[3,4.5] Surface plasmon resonance (SPR) was used to monitor dynamic mass accumulation on each type of surface. Zein adsorbed on both surfaces but accumulated faster and reached higher pile on -COOH than on $-CH_3$ surfaces.[4.5] It was proposed that zein rested on different sides of its prism-like molecule when adsorbed onto either hydrophobic or hydrophilic surfaces. The topography of zein deposits was examined by atomic force microscopy (AFM). On –COOH surfaces, large aggregates were observed, with grain size of 300 nm and roughness of 14.5 nm. On $-CH_3$ surfaces, zein deposited evenly with roughness down to 4.1 nm.

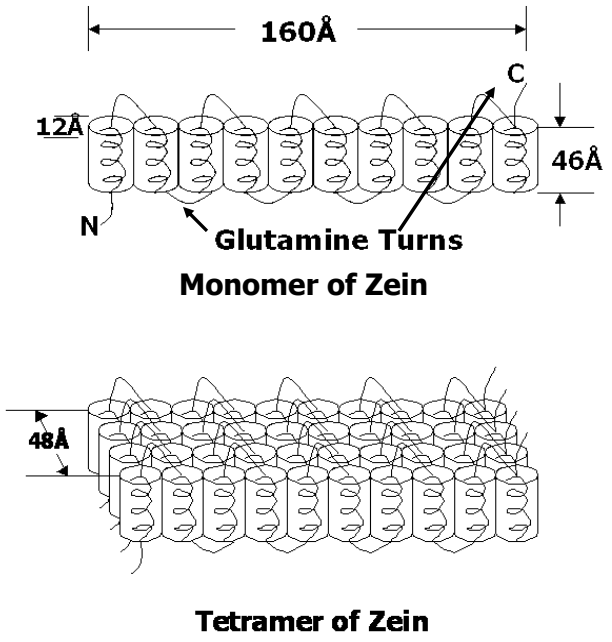


Figure 1. Zein monomer and tetramer structures [2].

These studies suggested that zein might be induced to form high pile or low pile layers upon adsorption, by selecting the appropriate substrates. The study also supported the notion that zein opposite surfaces are either hydrophobic (helix surfaces) or hydrophilic (top and bottom). The fact that zein can display different surface polar character depending on the self-assembly process can potentially be exploited to construct structures at the nano and microscale levels. This property is useful for tissue engineering since surface character is critical for cell attachment and growth[6]. Furthermore, zein properties may allow high precision in the construction of supra-molecular designs. Zein is biocompatible, reported to promote adhesion and cell proliferation.[7.8] Thus, our goal is to use zein for the construction of supramolecular structures utilizing nano and micro patterning techniques, namely dip-pen nanolithography (DPN) and micro-contact printing (μCP).

DPN is a scanning probe technique in which an AFM tip is used to deliver chemicals to a surface via a solvent meniscus, which naturally forms in the ambient atmosphere.[9] This direct-write technique offers high-resolution patterning capabilities for a number of molecular and biomolecular 'inks' on a

variety of substrates, such as metals and monolayer functionalized surfaces.[10] It was reported to generate nanoscale patterns of antibodies against the HIV-1 p24 antigen on gold surfaces.[11] It has also been used to create affinity templates for constructing arrays of nanoscopic features of active proteins.[12]

Micro-contact printing (μCP) is one of soft-lithography microfabrication techniques[13]. It has been widely used in micro-patterning thiols, proteins, and cells on different substrates including hard surfaces (i.e. gold, silver, metal oxide, glass) and soft materials (i.e. PLA, polyurethane). In our work, μCP was used to generate a pattern of hydrophilic surface on gold-coated glass followed by backfilling it with a hydrophobic thiol in order to provide a template to guide zein adsorption. Patterned zein surfaces were used as a secondary template for cell attachment and proliferation.

The first objective of this work was to study zein adsorption on nanopatterned surfaces of hybrid hydrophobic/hydrophilic surfaces generated by 16-mercaptohexadecanoic acid (MHA) and 1-octadecanethiol (ODT). The second objective was to explore the biocompatibility of zein surfaces self-assembled on micropatterned hybrid surfaces. Zein was adsorbed on uniform surfaces of carboxyl and methyl and inoculated with 3T3 fibroblast to test cell attachment and proliferation. Zein layers self-assembled on patterned surfaces containing hydrophilic and hydrophobic regions were studied for their ability to confine cell growth to selected areas.

Materials and Methods

DPN experiments

Nano-sized patterns were produced by DPN. Gold substrates (10 nm Cr and 60 nm Au on silicon wafers, Silicon Sense, Inc) were patterned with line arrays or grids consisting of monolayers of MHA (Aldrich, Milwaukee, WI). DPN patterning was done with a NSCRIPTOR™AFM (Nanoink, Inc., Chicago, IL) driven by custom lithography software (Nanoink, Inc., Chicago, IL). MHA-coated tips were prepared by dipping the single Si_3N_4 cantilevers (Type A, force constant = 0.05 N/m, Nanoink, Inc.) in an ethanol solution containing 5mM MHA for ~10 s. Contact-mode images were taken with the same NSCRIPTOR™ AFM. All DPN patterning experiments were conducted at 40% ± 1% relative humidity and 25 ± 1 °C. After patterning with MHA, wafers were immersed in an ODT (Aldrich, Milwaukee, WI) ethanol solution, 5 mM, for 1 hr, followed by rinsing with large quantities of ethanol. This procedure coated the unpatterned gold surface with a hydrophobic monolayer. After the ODT treatment, substrates were immersed in a zein solution (Showa Sangyo Co., Ltd, Japan, 0.4 mg/ml, 75% 2-propanol with 10 mM chloroacetic acid, pH = 3.7±0.3) for 1 hr, removed and rinsed with Nanopure water. Tapping-mode AFM (Asylum MFP-3D^{TM}, Asylum Research, Santa Barbara, CA) was used to image the topography of zein depositions.

μCP Pattern generation

Micro-sized patterns were generated by μCP, which has micrometer resolution and can be applied to large areas quickly. First, a pattern was designed using a computer based software (LEdit), then the pattern was printed on a transparency and transferred to a chromium and photoresistant (PR) - coated glass wafer through photolithography, which generates chromium mask. The pattern was transferred again to a glass wafer using photolithography. Such patterned wafer is used as mold. Finally, a rubber stamp with features in the geometry of those in the mold was made by casting and curing (at 90°C for 1 hr) a prepolymer of polydimethylsiloxane (PDMS) (Dow Corning, Sylgard 184) against the PDMS mold. After the stamp was released from its mold, it was rinsed by acetone, 2-propanol, and ethanol successively. The inked PDMS stamps were used to generate patterns on gold surfaces.

Sample preparation

Zein (Showa Sangyo Co., Ltd, Japan) solutions with a concentration of 3.0 mg/ml were prepared by dissolving zein in 75% 2-propanol with 10mM chloroacetic acid (pH = 3.7±0.3). Gold substrates were prepared by evaporating 3 nm Cr as adhesive layer and then 15 nm Au on 0.4 mm thick glass slides which were cleaned with Piranha solution right before the evaporation. Gold substrates were submerged into 2mM ethanol solutions of either MHA or ODT (both purchased from Aldrich, Milwaukee, WI) for 30 minutes to prepare carboxyl and methyl monolayers surfaces respectively. MHA or ODT-coated substrates were immersed in zein solutions (3.0 mg/ml, 75% 2-propanol with 10 mM chloroacetic acid, pH = 3.7±0.3) for 1 hour and then rinsed with Nanopure water.

Small pieces of PDMS for contact inking were prepared by placing the patterned stamp on the inked pad (PDMS flat stamp, immersed in a 2mM of MHA for at least 12 hr) without applying pressure for 40 s. During this process, conformal contact occurred between the two PDMS blocks, allowing transfer of thiols from one to the other[14]. A patterned MHA monolayer was generated simply by bringing the inked rubber stamp into contact with the gold for 30 seconds. Then, the patterned substrates were backfilled with 2mM ODT ethanol solution by leaving them in the solution for 30 minutes. Zein surfaces were generated by submerging the hybrid substrates into zein solution for 1 hr, which is then removed and rinsed with Nanopure water. To obtain the image of an MHA pattern, the patterned substrate before backfilling with ODT was etched by a gold-etchant (20mM $Fe_2(NO_3)_3$, 30mM Thiourea, water at ratio of 1:1:1), which removed the only gold not areas protected by the printed monolayer of MHA. The pattern was visible through an optical microscpe.

Cell culture

Mouse embryonic fibroblast cells (NIH 3T3) were purchased from American Type Culture Collection (ATCC) (Manassas, VA). The cells were maintained at 37°C, 5% CO_2 in a humidified incubator and were passaged every 3 days. The substrates with different surface treatments were placed in cell culture dishes. A glass ring was placed on top of each substrate to confine cells growing in a small area. Dulbecco's modified Eagle's medium (DMEM) supplemented with 4mM L-Glutamine, 4500mg/L Glucose, 10% fetal bovine serum (FBS), penicillin/streptomycin and 1mM sodium pyruvate was used as cell culture medium. All reagents were HyQ® brand and purchased from Hyclone (Logan, UT). Cells were plated at a density of 1 x 10^6 cells/mL onto the center of patterned surfaces in a region secured by glass ring which can contain up to 2 ml media. Thus prepared samples were cultured at 37°C, 5% CO_2 in a humidified incubator.

The morphology of NIH-3T3 cells growing on a cell culture dish, gold-coated glass slides, carboxyl monolayer, methyl monolayer, zein deposited on carboxyl, zein deposited on methyl, and zein deposited on patterned carboxyl and methyl surfaces were observed under phase contrast optical microscope (CK400, Olympus, Center Valley, PA) with images taken 72 hr after cell seeding. Cell morphology was determined by cell distribution and mean cell aspect ratio, with fibroblastic cells having an aspect ratio greater than 1.5[15].

Density of cells, 72 hr after incubation, was measured as follows. Before cells were inoculated, a glass ring greased with sterilized silicone vacuum grease was put on top of each substrate, which separated inside from outside surfaces permanently. Cells were only seeded in the ring and incubated in conditions described above for 72 hr. Mature cells were trypsinized, dyed with trypan blue (0.4%), and counted using hemocytometer. Cell density was expressed in percentage of control (% = counted number of samples / number of control).

Results and Discussion

Zein adsorption on nanopatterned surfaces

Zein binding to patterned MHA was confirmed by AFM. The image in **Figure 2** revealed that zein particles adsorbed and accumulated predominantly on MHA lines. The height profile shows raised features with an apparent height of 15 ± 1.3 nm which may be attributed to aggregation of zein molecules. Hydrophilic glutamine residues on top and bottom surfaces of zein molecules were thought responsible for zein binding to the -COOH groups of MHA. Hydrophobic aggregation of zein was promoted by addition of nanopure water. Zein deposited on ODT was uniformly distributed with surface roughness of 1.2 nm. Grain size measured 20 nm, which is very close to the length of a zein molecule. The MHA grid shown in **Figure 3** (850 nm × 850 nm) was also patterned by DPN. Section analysis shows zein accumulation on –COOH surfaces with a height of 27±5 nm. The height variation might be due to a non-uniform inking of MHA patterns or to zein impurities.

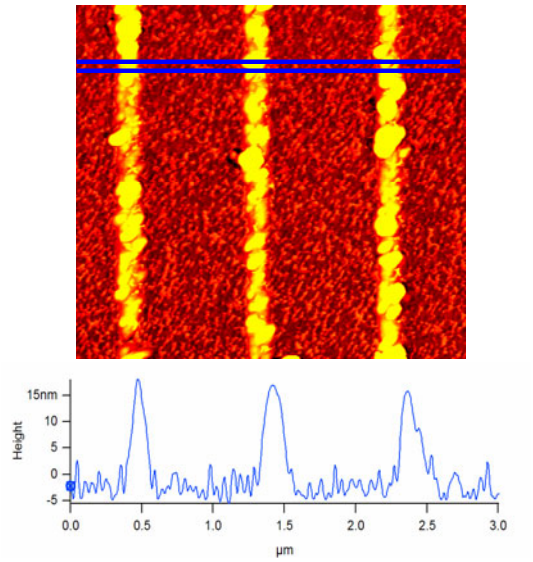
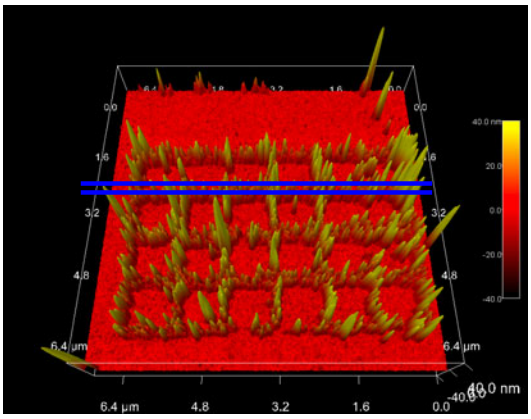


Figure 2. AFM image of zein on patterned MHA lines and corresponding height profile.



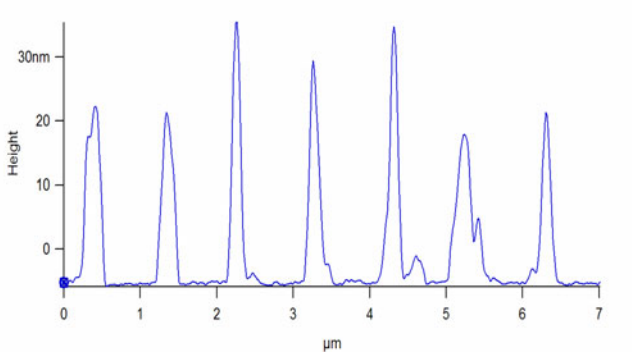


Figure 3. AFM image of zein on nanopatterned MHA grid and corresponding height profile.

Cell growing on uniform zein surfaces

NIH 3T3 cells grown on cell culture dish (control) after 72 hr of incubation are shown in **Figure 4**. Cells looked healthy and tended to stretch to adopt a spindle shape. They also show extensions that form a web-like network. Culture dish surfaces of are plasma treated and hydrophilic, known to promote cell attachment and proliferation. The aspect ratio of cells was around 6.5, suggesting that cells are healthy.

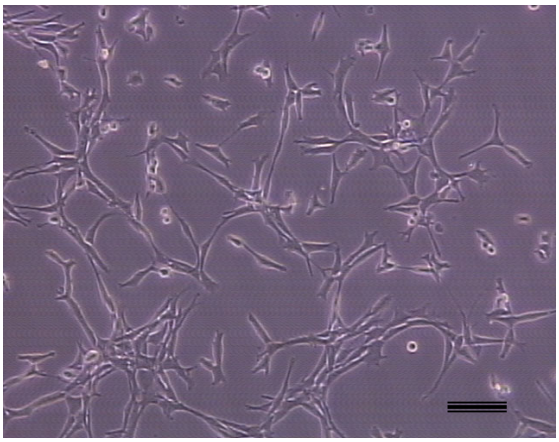


Figure 4. Cell growth on cell culture dishes after 72 hr of incubation, scale bar is 100μm.

The zein molecule contains both polar and non-polar amino acids. Its secondary structure has the polar domains and nonpolar domains located on two groups of surfaces, either parallel or normal to α-helices[2]. This arrangement creates surfaces with different polar character. This duality of surface character allows it to be manipulated into position by interaction with appropriate surfaces. It was concluded from SPR experiments that zein could interact with both carboxyl and methyl surfaces. However, it accumulated twice as much mass on carboxyl surfaces than that on methyl surfaces[3]. Moreover, zein deposits showed different height and morphology upon adsorption onto surfaces of different polar character or hybrid surfaces[3,5]. On carboxylic surfaces, zein piled up to form high clusters, while on methyl surfaces, zein laid down and formed low layer of coatings as illustrated in the DPN experiment section. These studies highlighted zein unique surface properties, which might favor cells to grow on selected surfaces. Therefore, in this paper, the surfaces of zein adsorbed on carboxyl and methyl surfaces were investigated for their biocompatibility by seeding fibroblast on them and incubating for 72 hr. The results show that surfaces of zein adsorbed on carboxyl surfaces gave a microenvironment which favored fibroblast to thrive (**Figure 5A**). Cell population was comparable to the control. Morphology was slightly different, cells had a tendency to form clusters, pointing out possible zein aggregation underneath. These observations are consistent with reports by Dong and coworkers[8]. They found that fibroblasts grew normally on zein films.

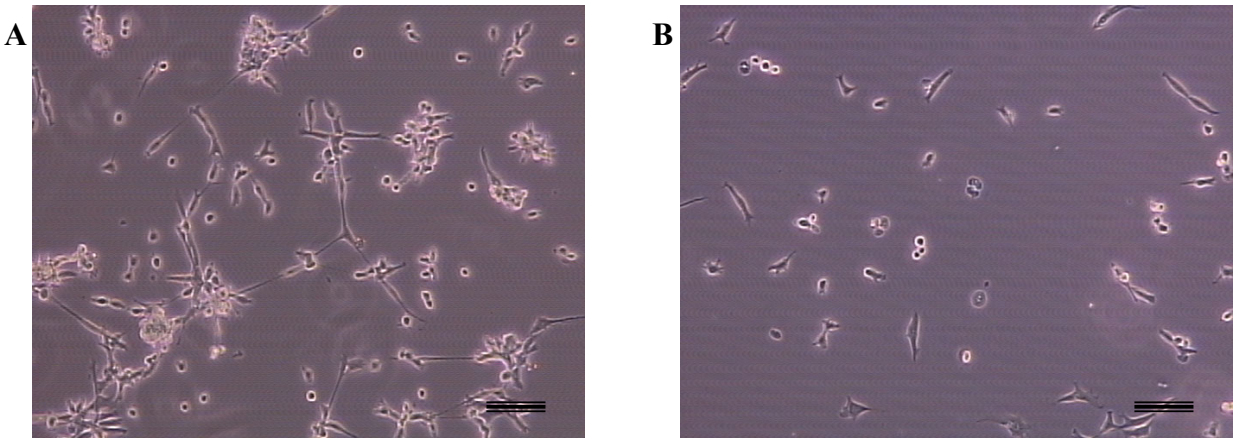


Figure 4. Cell growth on zein deposited on A) MHA, B) ODT surfaces, scale bar is 100μm

On zein adsorbed to hydrophobic surfaces, inoculated cells did not seem to attach or spread. Cells looked round and did not form extensions (**Figure 5B**). One possible reason is that zein used its

bottom hydrophobic side to interact with methyl monolayers, exposing the top hydrophobic side to the cells. Hydrophobic surfaces are reported to be unfavorable to fibroblast attachment and growth[16]. Another reason might be that protein mass accumulation was not high enough to cover all methyl surfaces so that cells lied on hydrophobic methyl groups rather than on zein molecules. It is well recognized that the hydrophilicity of extracellular matrix can influence morphological and functional properties of skeleton cells[6]. Fibroblast favors hydrophilic and smooth surfaces[16]. This phenomenon of cell population and morphology difference suggested zein surfaces formed on MHA are hydrophilic and hydrophobic on ODT. Several studies on the effect of surface polarity pointed out that cells (i.e. human osteoblasts, human bone derived cells) were capable to attach and grow on hydrophilic surfaces, however, they had difficulty of attaching and proliferating on hydrophobic surfaces[16,17].

Cell growing on patterned zein surfaces

Using μCP techniques, the monolayer of MHA successfully formed micropatterns on gold substrates with good edge definition, as shown in **Figure 6**. The line width was 25 μm and line to line distance was 100 μm. Contact inking, described in the methods section, was chosen to generate clearly defined pattern since it prevents distortion of the pattern by swelling and also reduces crystallization of thiols on the surface of the inked stamp[14]. Falconnet et al.[18] offered a comprehensive review on surface engineering approaches to micropattern surfaces for cell-based assays. In their paper, they considered that μCP is the most widely used among other soft lithography techniques because of its simple, cost-effective, and flexible attributes.

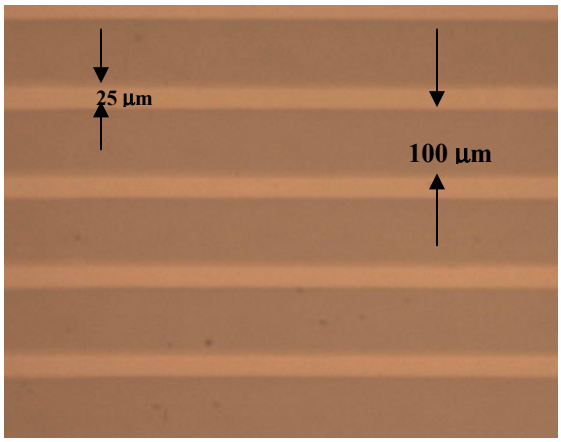


Figure 6. Micropattern of MHA lines made by micro-contact printing

Zein hybrid surfaces were generated via self-assembly of zein molecules on a hybrid template with patterned hydrophilic/hydrophobic surfaces produced by micro-contact printing. To demonstrate the efficacy of patterned zein surfaces on spatially controlling cell growth, 3T3 fibroblasts were seeded onto these surfaces and their growth and morphology were observed. Since only the lines were covered with carboxyl monolayers which the cells can attach to and grow, and most of surfaces were covered with methyl monolayers, which does not support cell growth, cell density was low and it took about 5-6 days for cells to pass lag phase and grow faster in the log phase. This extended lag phase could also be attributed to the increased roughness of surfaces, which has been discussed by Wang[4,5]. At sixth and eleventh days after inoculation, fibroblast growth was observed following the patterned lines. The patterned cells are shown in **Figures 7A** and **7B**. Those images suggested cells had high preference to attach and spread exclusively on patterned zein on MHA surfaces. Right after inoculation, cells attached and grew where they landed. As time went on, they had the tendency to move to more favorable places.

The elongated fibroblasts had a width around 15-20 μm and aspect ratio around 14.5, suggesting that the cells were thriving on the hydrophilic surfaces underneath.

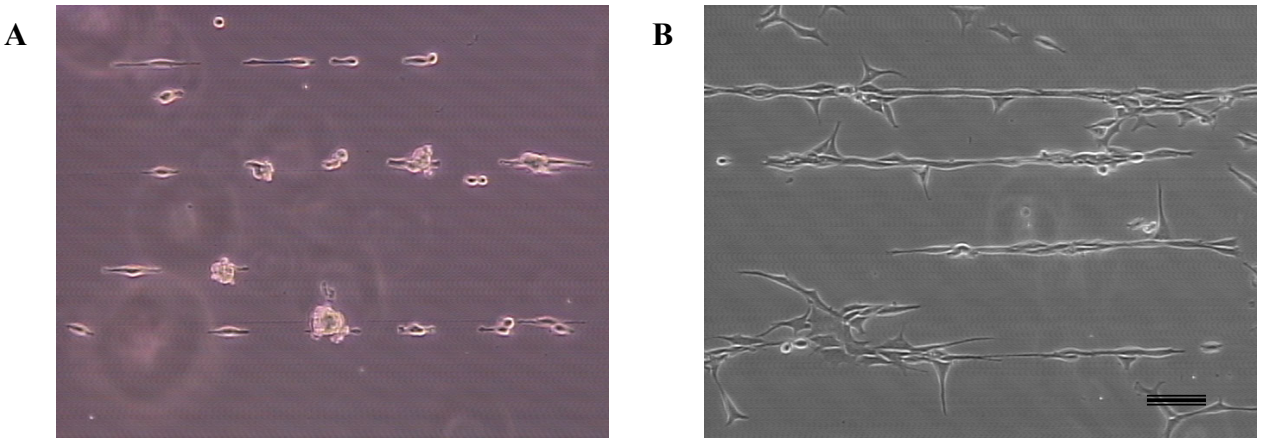


Figure 7. Cell growth on zein patterns after **A**) 6 days and **B**) 11 days of incubation, scale bar is 100μm.

Several papers have been published on cell growth on patterned surfaces. Nam et al.[19] studied neuron cell attachment on proteins in order to detect electrical impulses from brain. Fibroblasts were patterned on poly(lactic acid) (PLA) and poly(lactide-*co*-glycolide) PLGA films for controlling the spatial morphology and distribution of cells by Lin et al.[20] Our results clearly show that cells can be patterned on a zein-coated surface by taking advantage of its dual polar character surface.

Zein has been studied for its mechanical properties, biodegradability and biocompatibility, serving as a biomaterial for tissue scaffold in regenerative medicine or control release matrix in recent years. Results show that zein not only has good mechanical and manufacturing properties, but also is biocompatible in terms of cell attachment, growth, and proliferation.[7,8] It was also reported that the pepsinization half-life of zein was less than 2 weeks (Gong 2006), suggesting that zein is readily biodegradable. Our work suggested that zein can be manipulated based on its hydrophobic/hydrophilic character to guide and confine cells to grow in selected areas. Zein could be used effectively to control spatial location, orientation, and morphology of seeded cells. A controlled microenvironment is considered critical in the development of engineered tissues. Future work is needed to generate micropatterned surfaces on soft materials to form 3D structures either by layer-by-layer construction or other enabling technologies.

Conclusions

Zein nanoscale structures were built on MHA templates patterned on gold surfaces by DPN. Zein shows differential adsorption between MHA and ODT, used as a background ink. Zein molecules formed high rise structures on MHA surfaces and low laying deposits on ODT. Topographical features of zein deposits, as observed by AFM were clearly defined owed to zein self-assembly properties and precision nanolithography. The controlled growth of fibroblast on patterned zein surfaces was also demonstrated. Zein adsorbed to carboxyl surfaces favored cells to attach and proliferate. In contrast, zein adsorbed to methyl surfaces limited cell growth. Micropatterned surfaces having hybrid hydrophilic and hydrophobic properties were generated by micro-contact printing of MHA and back filling the unpatterned surfaces with ODT. Zein self-assembly on patterned surfaces generated another template, which was used for growing fibroblast. Results show that cells seeded on patterned zein surfaces were

confined to the area of zein on carboxyl groups. Thus made zein structures would be useful in controlling the spatial organization of cells on tissue scaffolds.

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