CHAPTER III

Generation of Surface-Bound Multicomponent Gradients

Abstract

Cell migration and differentiation are regulated by soluble or matrix-bound gradients in biological systems. Usually, multiple signals are involved in these processes. Herein, we have developed a new method for the generation of protein films with precise control over the spatial density of several different proteins. First, an artificial polypeptide scaffold composed of separate protein capture and surface anchor domains was designed and synthesized. These domains contain a heterodimeric leucine zipper system and an elastin mimetic motif. By using a mutant *E. coli* phenylalanyl-tRNA synthetase, the photoreactive amino acid *para*-azidophenylalanine was incorporated. Glass slides were then functionalized with this polypeptide by spin coating and photocrosslinking. The resulting protein-coated slides were subsequently secured underneath microfluidic chips specifically designed for on-chip mixing using laminar flow. Gradients of leucine zipper tagged proteins were formed in the microchannels and immobilized on the engineered protein films through association of the coiled-coil heterodimer. The dissociation kinetics of the immobilized proteins was also investigated. Lastly, the adhesion of human umbilical vein endothelial cells cultured on the surface-bound gradients of cell binding ligands generated by this technique were examined.

3.1 Introduction

In biological systems, gradients of soluble or matrix-bound stimuli are crucial to many important cellular processes. For instance, under the guidance of specific signaling protein gradients, stems cells move to the right positions to form organs,[1] axons extend their growth cones over long distances to establish the correct synaptic connections,[2] and endothelial cells migrate into surrounding tissues to initiate angiogenesis.[3] In addition to directing cell migration, gradients play important roles in embryonic development, where the spatial and temporal presentation of morphogens specifies cell fate and results in the formation of patterned tissues.[4] Therefore, it is of general interest to engineer microenvironments with a spatially controlled presentation of extracellular cues either for studying gradient-sensing mechanisms *in vitro*, or for developing instructive materials that elicit desired cellular responses.

One of the most widely used techniques to study chemotaxis is the Boyden chamber assay.[5] Chemotatic gradients are generated by placing different concentrations of chemo-attractants in the upper and lower compartments separated by a porous filter through which cells can migrate. This assay does not allow direct visualization of cell locomotion, and gradient shapes are not well defined.[6] To produce gradients on the biological length scale with single-cell resolution (10-100 μm), advanced techniques such as microfabrication are needed. Recently, Jeon et al. proposed the design of a microfluidic gradient generator that harnessed laminar flow of fluids in poly(dimethylsiloxane) (PDMS) channels to create concentration

gradients of molecules in solution.[7] From a materials design perspective, it is more practical to generate surface-bound rather than solution-phase gradients because smaller volumes of reagents are consumed during the experiments, and the modified materials may be readily used for tissue engineering applications. Currently, there are two major ways to immobilize protein gradients on material surfaces. Nonspecific adsorption is the simplest way to produce such gradients,[8,9] but the resulting structures are quite unstable due to fast surface dissociation kinetics[10] and cell remodeling[11]. Alternatively, immobilization via covalent crosslinking through lysine and cysteine residues provides increased stability of surface-bound gradients.[10] Unfortunately, both methods result in the random orientation and altered conformation of proteins, and the biological activities of proteins are greatly reduced. Moreover, these methods are not generally applicable to generating multi-ligand gradients that usually encountered *in vivo* by cells since some proteins (such as small peptides) have limited surface-exposed reactive residues for conjugation or are too hydrophilic to adsorb stably onto hydrophobic substrates.

These difficulties can be overcome by engineering site specific attachment of ligands through expression of recombinant fusion proteins bearing affinity tags. Previously, we designed an artificial polypeptide scaffold for protein immobilization onto solid substrates.[12] By combining this surface functionalization method with microfluidics technology, we developed a new approach to generate surface-bound multicomponent protein gradients.

3.2 Materials and methods

3.2.1 Cloning, expression and purification of proteins

The genes encoding the elastin mimetic domain ELF and the heterodimeric leucine zipper pair, ZE and ZR, have been previously constructed.[12] Since ZRELF is more soluble than $ZE(gs)_6ELF$, we use this newly designed protein for surface functionalization. ZRELF containing the photoreactive unnatural amino acid, *para*-azidophenylalanine was biosynthesized using the phenylalanine auxotroph *E. coli* strain AF-IQ.[13] Two proteins, a mutant tenth fibronectin type III domain[14] (FN) with specificity to $\alpha_v\beta_3$ and a *de novo* designed VEGF mimetic peptide[15] (QK), were selected as the model ligands for gradient generation and their genes were obtained by assembling synthetic oligonucleotides through PCR. Recombinant proteins FNZE, QKZE and ZE were expressed in *E. coli* strain BL-21 at 22 ^oC. All proteins were purified on Ni-NTA columns and their purity was assessed by SDS-PAGE and MALDI-TOF mass spectrometry.

3.2.2 Surface functionalization

Standard glass slides (Corning) were immersed into concentrated H_2SO_4 for one hour. After washing thoroughly with water, they were immersed into a boiling solution of 1/1/5 (v/v) H_2O_2 (30%) /NH_4OH (30%)/H_2O for 30 minutes. Then the slides were gently shaken in 1% octyltrichlorosilane in toluene for three hours. Finally they were cleaned twice in methanol and twice in DI water. The functionalized slides

were cured at 110^{o}C for 30 minutes.

A solution of ZRELF (50 µl, 2.5 mg/ml) in 50% n-propanol was applied to a small part of a glass slide and spun at 1,500 rpm for 45 seconds. The resulting protein film was irradiated with Hg-arc lamps (I-line and H-line, 4.5 mW cm^{-2}) in a Karl Suss mask aligner for 2 minutes. Exposed protein-coated slides were thoroughly washed with 50% isopropanol and ddH_2O to remove uncrosslinked protein.

3.2.3 Fabrication of microfluidic chips

The microfluidic gradient generator was fabricated using rapid prototyping and soft lithography as originally described by Jeon et al.[7] Briefly, a high-resolution printer was used to generate a mask with a minimum feature size of 30 µm from a CAD file (CAD/Art Services, Poway, CA). A SU-8 2100 photoresist (Microchem, Newton, MA) layer was spin-coated onto a silicon wafer at 3500 rpm for 30 seconds and exposed to ultraviolet light for 150 seconds through the mask with the pre-printed pattern in a Karl Suss mask aligner. The wafer was then immersed into SU-8 developer, and the unexposed photoresist was dissolved into solution, leaving behind a master mold composed of 100-µm high crosslinked photoresist structures. Poly(dimethylsiloxane) (PDMS) chips were formed by curing prepolymer solution (Sylgard 184, Dow Corning) on silicon masters. Inlet and outlet ports were punched out of the PDMS using a sharpened needle. Polyethylene tubing was inserted into these holes to enable fluid flow in and out of the microchannels.

3.2.4 Quantitative measurement of surface density

Lyophilized samples of FNZE or QKZE (1 mg each) were dissolved in 0.5 ml of $NaHCO_3$ buffer (100 mM, pH~9, adding SDS until dissolution) and reacted with 0.5 mg of Cy3 (Amersham) or Alexa 647 (Invitrogen) NHS ester for 5 hours at room temperature. Dialysis was used to remove unconjugated dye molecules. Then the dialyzed samples were subjected to gel electrophoresis, and the ratio of conjugated to unconjugated dye molecules was determined by fluorescence imaging. Protein solutions (2 μl each) at different concentrations (0.2, 0.1 and 0.05μM of FNZE; 0.4, 0.2 and 0.1μM of QKZE) were spotted onto ZRELF-coated glass slides and dried in air over night. The fluorescence intensity and the area of each spot were measured by a GenePix 4200A chip reader. The surface density at each spot was calculated and plotted against fluorescence intensity. The final curves were linearly fitted with intercepts set to zero (the scanning setting was adjusted to minimize the background fluorescence to zero arbitrary unit).

3.2.5 Determination of dissociation kinetics

FNZE and QKZE solutions (0.5 μM) were spotted onto ZRELF-coated glass slides and incubated for one hour. The slides were then sonicated in an excess of PBS for half an hour. Keeping the samples hydrated, the slides were transferred to glass beakers containing 50 ml PBS or Dulbecco's modified Eagle's cell culture medium (CO_2-independent pH equilibration, Invitrogen). The beakers were placed in a 37 ^oC

incubator, and fluorescence intensities of immobilized proteins were measured over time using a Carl Zeiss microscope.

3.2.6 Cell culture

Human umbilical vein endothelial cells (HUVECs, Clonetics) were maintained in a 37 °C, 5% CO_2 humidified environmental chamber. The cells were grown in endothelial cell basal medium (EBM-2, Clonetics) supplemented with the supplied Bulletkit, which was replaced every 2 days. Near confluent HUVEC cultures were passaged nonenzymatically by treatment with 0.61 mM EDTA (Gibco). Passages 3–6 were used.

3.2.7 Generation of immobilized protein gradients

The PDMS chip and glass substrate (ZRELF-coated region was covered with a protection box[9]) were activated with oxygen plasma (200 millitorr, 35 sec, 80 W, Anatech) and coupled immediately to form an irreversible seal in order to prevent leakage when injecting fluids. To remove trapped bubbles and block nonspecific protein adsorption, microfluidic channels were flushed with 2% BSA solution for half an hour using a PHD 2000 syringe pump (Harvard Apparatus). Afterwards, relevant protein mixtures diluted in 2% BSA solutions were individually injected into the two inlets at a rate of 0.5 μl/min for one hour to generate immobilized protein gradients. The whole assembly was then soaked in water and the PDMS portion above the

gradient region was peeled away from the remaining chip using a razor blade. This resulted in the formation of a PDMS well on the glass slide. The sample was sonicated in PBS for half an hour and blown dry, and the gradient profile was scanned by a GenePix 4200A chip reader. For substrates used for cell studies, PBS was left in the PDMS well to maintain the hydrated gradient surfaces.

3.2.8 Cell studies

The gradient region containing the PDMS well was cut out from the glass slide using a diamond pen and placed into a 6-well tissue culture plate. The whole set was sterilized upon ultraviolet light exposure for five minutes in a laminar flow hood. Passaged HUVECs resuspended in 5 ml serum-free EBM-2 containing 2% BSA were added to the samples at a density of 10,000 cells /cm^2. After 2 hours, the plates were removed from the incubation chamber, gently washed twice with EBM-2 containing 2% BSA, and imaged using a 10× phase contrast objective on a Nikon Eclipse TE 300 inverted microscope. Images were captured on a Sony CCD color video camera (model DXC-151A) equipped with Metamorph software. Fifteen images randomly taken from three gradient substrates were used to quantify the cell attachment.

3.3 Results and discussion

3.3.1 Design and biosynthesis of relevant proteins

The ZE/ZR heterodimer leucine zipper pair was derived from vitellogenin-binding protein (VBP), a bZIP homodimer.[16,17] All residues at the **e** and **g**

positions of the first four heptads were changed to glutamic acid or arginine, respectively, to produce acidic peptide ZE and basic peptide ZR.[12] In our hands, ZE can be stably expressed in *E. coli*, while ZR cannot be produced unless it is fused to a well-folded protein or co-translated with ZE. Therefore, ZE was chosen as a universal affinity tag to make recombinant FNZE and QKZE fusion proteins. Meanwhile, ZR was fused to elastin mimetic domain ELF to make ZRELF peptide for surface functionalization. ZRELF was co-expressed with ZE using a dicistronic construct as described previously.[12] After purification, dialysis and lyophilization, the protein yields of ZRELF, QKZE, FNZE and ZE were 75 mg, 80 mg, 150 mg and 30 mg per liter of culture, respectively. Sample purity was analyzed by SDS-PAGE (Figure 3.1). According to amino acid analysis, the incorporation rate of *p*-azidophenylalanine in ZRELF is 40%.

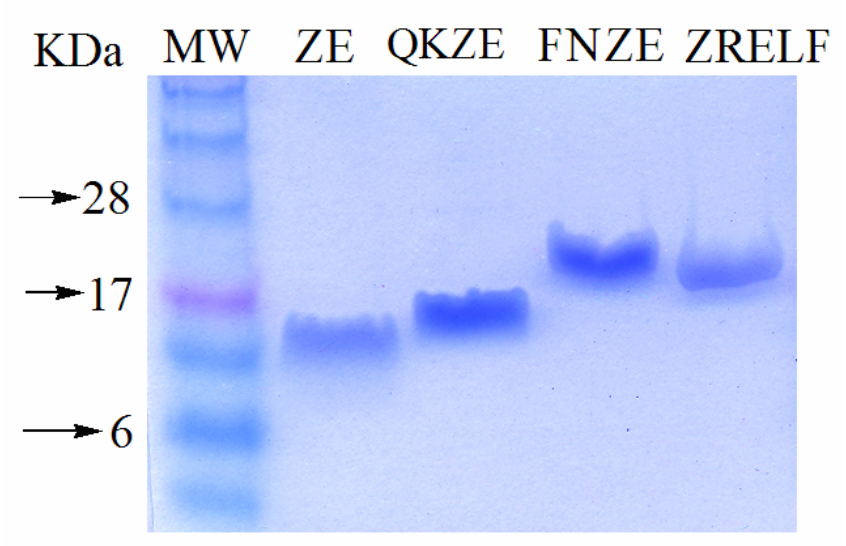
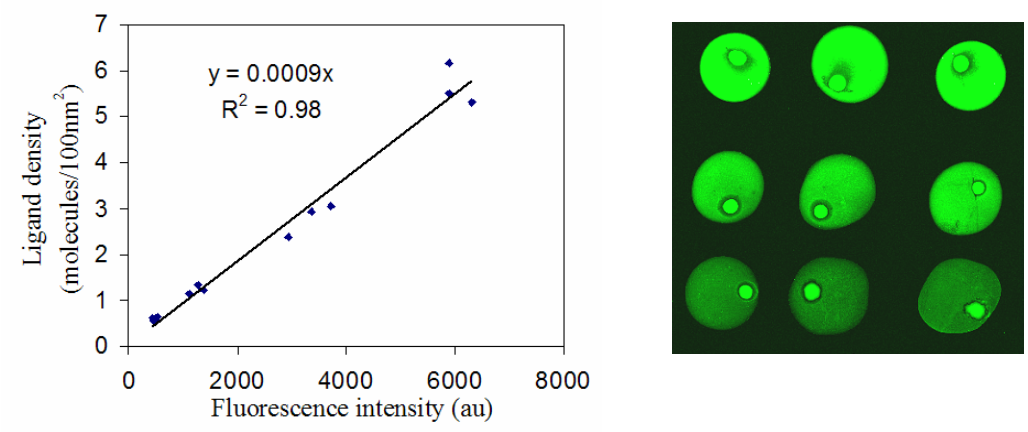


Figure 3.1 SDS-PAGE of purified proteins.

3.3.2 Generation and characterization of immobilized protein gradients

To quantify the surface density of proteins, we labeled FNZE and QKZE with Cy3 and Alexa 647 fluorophores, respectively. Aliquots at different protein concentrations were spotted onto the surface of ZRELF-coated glass slides and dried (right panel of Figure 3.2). After measuring the fluorescence intensity and area of

(a)



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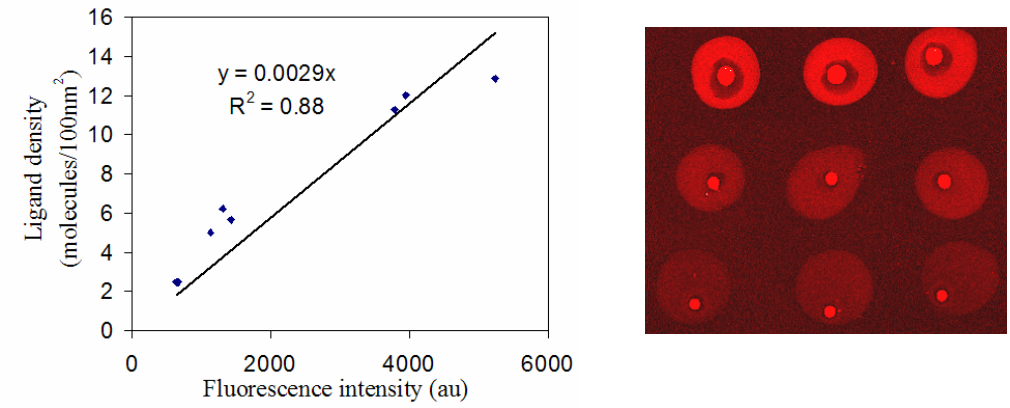
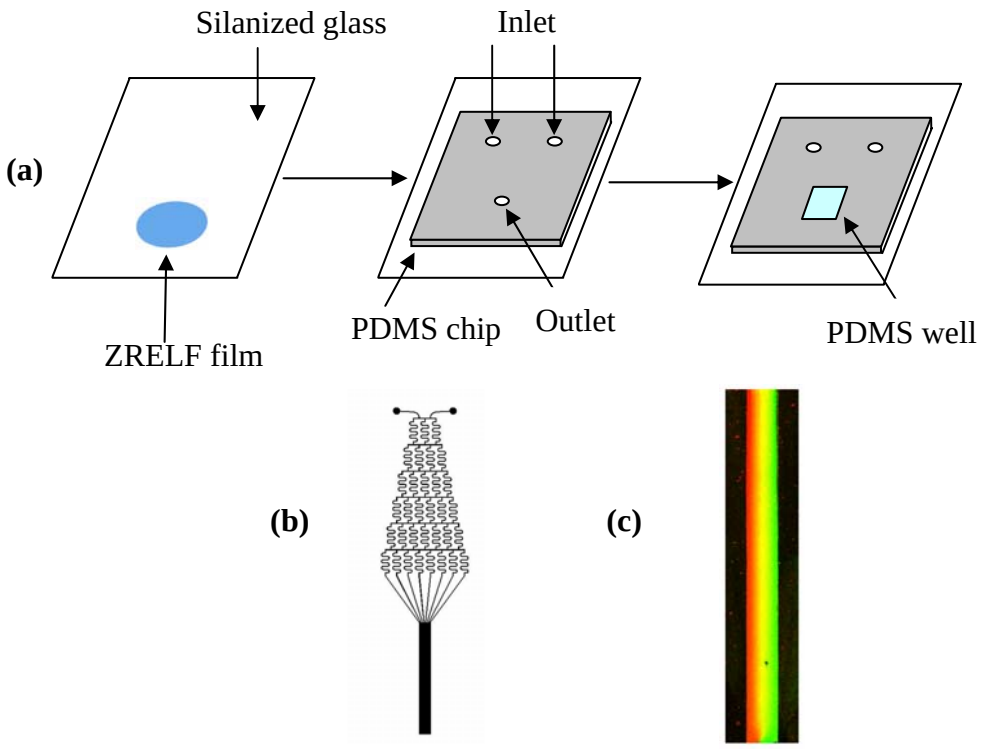


Figure 3.2 Calibration curve for quantification of ligand density. (a) Cy3 conjugated FNZE, (b) Alexa 647 conjugated QKZE. Protein solutions (2 μl) were spotted and dried. The fluorescence intensity and the area of each spot were measured by a GenePix 4200A chip reader. The final curves were linearly fitted.

these spots, standard curves correlating surface density (molecules/100nm^2) and pixel intensity (au) were constructed (left panel of Figure 3.2). Linear relationships were obtained when the protein solutions were spotted on ZRELF films since strong coiled-coil association allowed homogenous distribution of protein molecules during drying. If blank glass slides were used, most of the protein molecules were concentrated in the center of the drying spots and the linear relationship was lost. Calibration curves from linear regression were used to estimate the surface concentration of immobilized protein gradients.

As illustrated in Scheme 3.1a, gradients of surface-bound protein ligands were obtained by combining surface functionalization and microfluidic delivery. Glass slides pretreated with OTS were spin-coated with a solution of ZRELF dissolved in 50% propanol (the addition of organic solvent helped wet the hydrophobic surface). The protein films were covalently crosslinked to the substrates through photodecomposition of the arylazides upon UV irradiation. A microfluidic gradient generator (Scheme 3.1b) fabricated from PDMS was sealed to the functionalized glass slide after oxygen plasma activation. Different solutions containing ZE tagged proteins were continuously injected into the microfluidic network using a syringe pump. The fluid streams introduced through the two inlets were combined and mixed in the serpentine channels and eventually established a concentration gradient across the output channel. There the protein gradients from solution were immobilized on the ZRELF film coated underneath the microchannel

via coiled-coil association between ZE and ZR. After generation of surface-bound gradients, the device was soaked in water and the PDMS portion above the gradient region was gently removed. The resulting well kept the proteins in solution (drying will denature proteins and remove bound proteins from surface) and facilitated studies of cells to be cultured on this surface. Scheme 3.1c shows a fluorescence image representing the immobilized counter-gradient of QKZE and FNZE across the entire output channels (800 μm wide and 1 mm long).



Scheme 3.1 The assembly of microfludic device and gradient generation. (a) Steps to assemble the setup to generate gradients. (b) Schematic design of the gradient generator. (c) Fluorescent image of the counter-gradient of QKZE and FNZE across the output channel.

FN (94 residues) and QK (15 residues) were chosen as model proteins and C-terminally fused with the affinity tag ZE to generate FNZE and QKZE recombinant proteins. The ZE peptide was also required as an "inert" density controller to adjust the available binding sites. To form smooth linear gradients, a flow rate of 0.5 μl/min and a binding period of 1 hour were empirically determined. First, we generated a surface-bound gradient of FNZE by injecting ZE and FNZE (both at a concentration of 100 nM) through the two inlets. In the microfluidic network, solution with increasing concentrations of FNZE was accompanied by decreasing concentrations of ZE. Both proteins competed for the same binding sites on the surface, thus the gradient in solution was translated into the immobilized gradient. Based on the calibration curve (Figure 3.2) and fluorescence image of the gradient (left panel of Figure 3.3a), the corresponding density profile of FNZE was obtained (right panel of Figure 3.3a). For these substrates, the protein density varied from zero to 4.5 molecules/100nm^2 across the 800 μm channel with a slope of 56.25 molecules/μm^3.

To investigate the possibility of generating gradients containing multiple species, QKZE was mixed with FNZE and both were introduced through the same inlet. A solution of ZE was introduced through the other inlet. All protein solutions were at the same concentration (100 nM). As shown in Figure 3.3b, an overlapping gradient of FNZE and QKZE was created. The density of FNZE was increased from zero to 1.8 molecules/100nm^2 across the channel, while that of QKZE was increased from zero to 6.4 molecules/100nm^2. This result demonstrated that even though FNZE

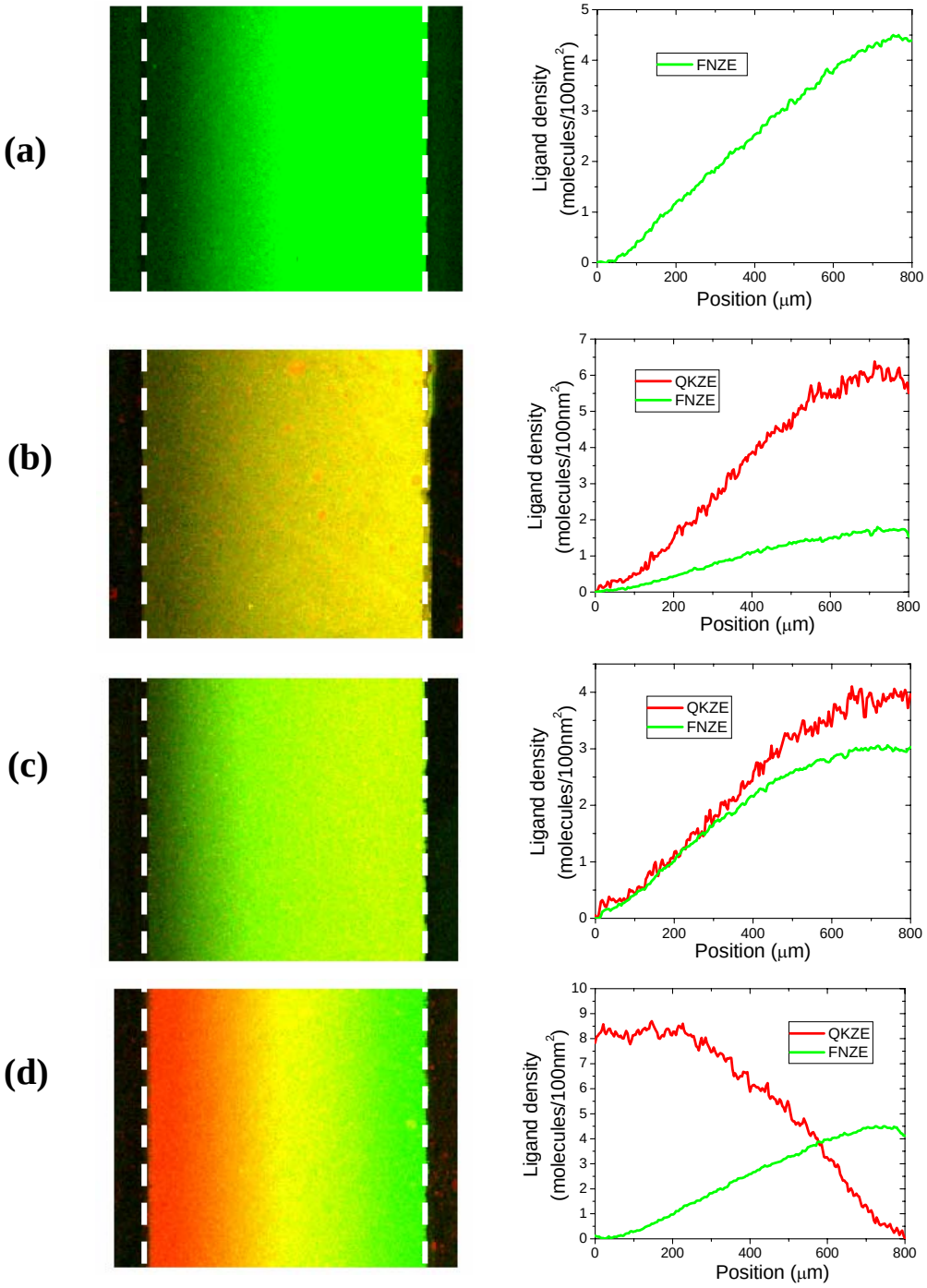


Figure 3.3 Fluorescence images of immobilized gradients and their corresponding density profiles. Cy3 (FNZE) was pseudocolored green, Alexa 647(QKZE) red and overlay yellow. (a) ZE(100 nM) ↔ FNZE(100 nM); (b) ZE(100 nM) ↔ FNZE(100 nM), QKZE(100 nM); (c) ZE(100 nM) ↔ FNZE(100 nM), QKZE(50 nM); (d) QKZE(100 nM) ↔ FNZE(100 nM).

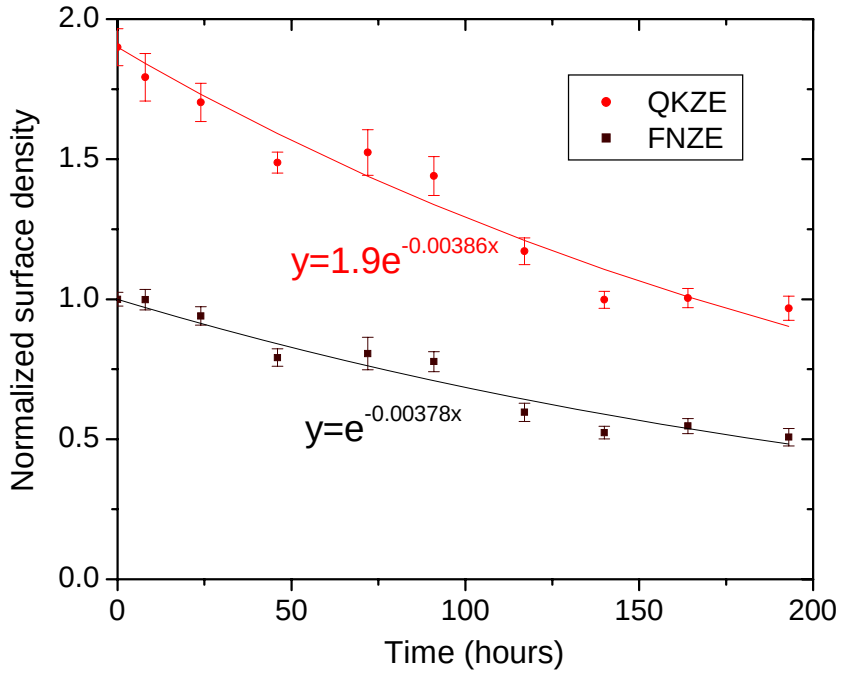
and QKZE were at the same concentration in the solution, the resulting surface densities were significantly different. A possible explanation for this result may be that since the size of QKZE is smaller than that of FNZE, QKZE has faster binding kinetics and occupies more sites per unit area because of less steric hindrance. The densities of each protein can be controlled by changing the ratio of FNZE to QKZE in the injection solution. For example, when the concentration of FNZE was maintained at 100 nM and the concentration of QKZE was reduced to 50 nM, a gradient with different profile was produced (Figure 3.3c). In this case, the density profile of FNZE spanned from zero to 3 molecules/100nm^2 compared to that of QKZE, which varied from zero to 4 molecules/100nm^2. We also generated immobilized counter-gradients as demonstrated in Figure 3.3d by inputting QKZE and FNZE from different inlets. The density of FNZE gradually decreased from 4.5 molecules/100nm^2 to zero while the density of QKZE increased from zero to 8.6 molecules/100nm^2.

3.3.3 Dissociation kinetics of immobilized proteins

For cell studies and tissue engineering, it is important to maintain the long-term stability of the surface characteristics of engineered materials. We soaked the protein-bound surfaces in excess buffer and monitored the dissociation kinetics through repeated fluorescence imaging. The desorption curves are shown in Figure 3.4. The density at time zero corresponds to the amount of proteins remaining on the surface after sonication in PBS buffer for half an hour, which is a critical step to

remove weakly bound molecules. When soaked in PBS, following the first-order kinetics law, the proteins gradually diffused away from the surface (Figure 3.4a). Both QKZE and FNZE had a very slow release rate. The dissociation rate constant k_{off} was around $1.1x10^{-6}$ s^{-1}, approximately two-fold lower than that observed for biotin/streptavidin complex ($2.4x10^{-6}$ s^{-1}).[18] This meant that half of the proteins still remained attached after 175 hours. Interestingly, QKZE and FNZE have very similar dissociation kinetics despite difference in both their molecular weights and three dimensional structures. The phenomenon indicates that after immobilization, surface dissociation mainly depends on the coiled-coil interaction between ZE and ZR, irrespective of their fusion proteins. However, these proteins dissociated faster in cell culture medium (Figure 3.4b). Dissociation of FNZE could still be described by the first-order rate law, and the rate constant was two-fold higher than that in PBS. QKZE showed an initial burst release in the first 24 hours. Beyond 24 hours, the dissociation of QKZE followed a slow first-order process comparable to that of FNZE. The faster dissociation in cell culture medium may relate to the higher ionic strength, which shields the electrostatic interactions between arginine and glutamic acid residues.[16]

(a)



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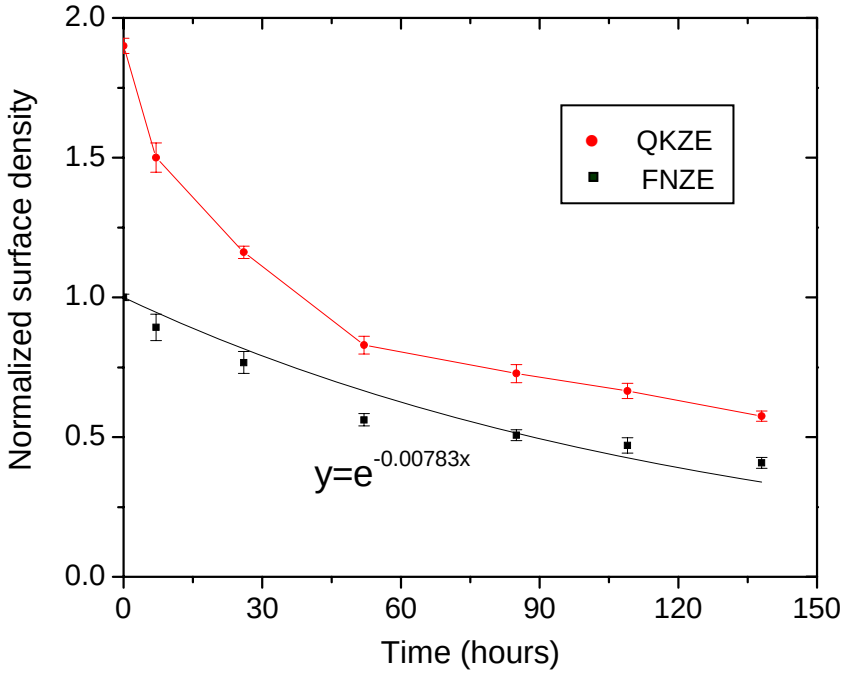

Figure 3.4 Dissociation curves of immobilized proteins monitored by changes in surface fluorescence. (a) Slides soaked in PBS. (b) Slides soaked in CO_2-independent Dulbecco's modified Eagle's cell culture medium.

3.3.4 Cellular response to surface-bound gradients

Finally, we subjected our substrates to cell studies. On a control substrate with only ZE peptide, very few HUVECs adhered, and these cells did not spread and showed a round morphology (Figure 3.5a). In contrast, when FNZE was immobilized on the surface, significant cell adhesion was observed (Figure 3.5b), demonstrating that cells recognized the adhesion ligand within the recombinant protein FNZE. The attached cells distributed homogeneously across the entire substrate, and most of them exhibited well-spread morphologies.

Alternatively, a gradient shown in Figure 3.3a had an increasing density of FNZE as well as a decreasing density of ZE across the substrate. To this surface HUVECs were seeded (Figure 3.5c), and as expected, the number of adherent cells varied spatially across the gradient. It was discovered that more cells attached to the region with a higher density of cell adhesion ligands. To quantify the cell attachment, each image (800 μm wide by 600 μm long) was split into three regions, and the fraction of adherent cells was determined through dividing the number of cells per region by the total number of cells attached (Figure 3.5d). On the left third of the gradient, the fraction of attachment was 0.13±0.05; the fraction increased to 0.36±0.06 on the middle third of the gradient; and on the right third, the fraction reached the highest value, 0.51±0.06.

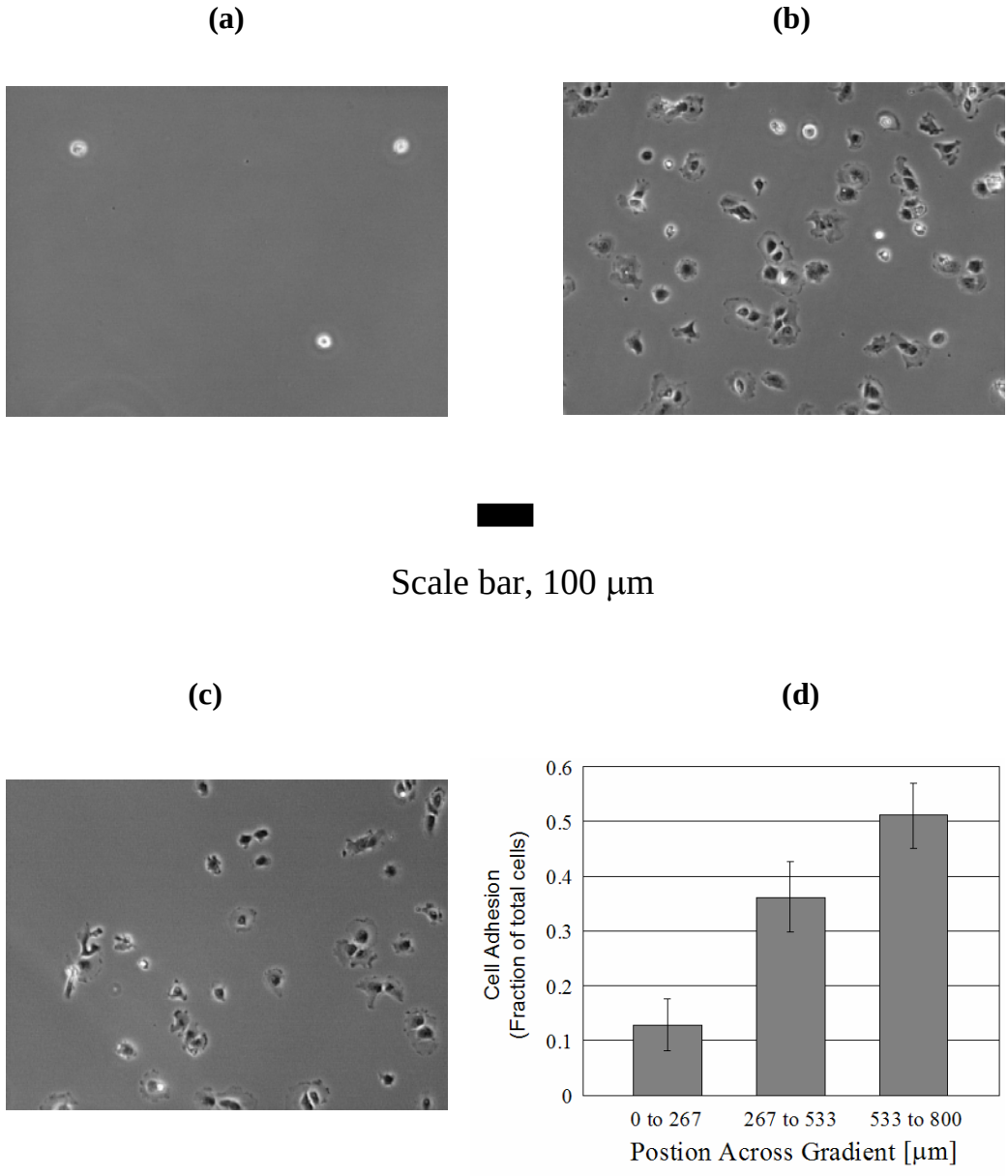

Figure 3.5 The attachment of HUVECs. Phase contrast images of HUVECs attached to the surface of (a) ZE, (b) FNZE, (c) ZE◂▸FNZE gradient. (d) Quantification of HUVECs adhesion.

3.4 Conclusion and future work

We have demonstrated that the integration of microfluidics, protein engineering and surface functionalization provides a simple and flexible approach to generating surface-bound multicomponent gradients. We have also showed that gradient of cell adhesion ligands generated by this technique modulates the attachment and spreading of endothelial cells. In principle, any combination of proteins or peptides can be introduced into this system and their cooperative effects on cellular response could be investigated. Moreover, the relative ratio of different proteins or steepness of gradients can be easily and precisely controlled. Since it is relatively straightforward to engineer leucine zippers with various affinities by changing the number of heptad repeats[17], the surface stability can be tuned for specific proteins, which may be harnessed to construct dynamic surfaces that allow spatiotemporal control of protein ligands. We can also incorporate this leucine zipper system into hydrogel-forming protein polymers[19] and the generation of gradients in such three-dimensional scaffolds may be used to guide cell behaviors such as neuron regeneration or angiogenesis.

3.5 References

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