CHAPTER IV

Progress towards the Creation of Protein Walkers

Abstract

The extracellular matrix serves many important functions such as maintaining the structural organization of tissues, providing support and anchorage for cells, and regulating cellular physiology. Cell-ECM interactions are mediated through specific cell surface receptors known as integrins. Clustering of integrins into focal adhesions plays a central role in determining cell behaviors. Our goal is to generate protein walkers that have different lateral mobility rates on a surface, which, if presenting cell adhesion ligands, may influence the clustering of integrin receptors. Herein, an artificial polypeptide scaffold was developed for protein immobilization onto solid substrates via heterodimeric coiled-coil association. Several variants of a leucine zipper pair that have tunable heterodimerization affinities were designed and synthesized to allow diversity in the association strength of proteins linked to a surface. Then four proteins containing either two or three of these binding motifs were created. However, FRAP experiments did not provide evidence of controlled lateral mobility of these proteins on a surface modified with the designed polypeptide scaffold.

4.1 Introduction

Most mammalian cells in tissues are surrounded by an insoluble network of fibrous proteins and polysaccharides that comprise the extracellular matrix (ECM).[1] The ECM serves many important functions such as maintaining the structural organization of tissues, providing support and anchorage for cells, and regulating cellular physiology.[2,3] The ability of cells to proliferate, differentiate, and migrate largely depends on their capacity to bind to certain components of the ECM. Cell-ECM interactions are mediated through specific cell surface receptors known as integrins. An integrin is a transmembrane heterodimeric complex composed of noncovalently associated α and β subunits. Upon binding to recognition sites (such as the Arg-Gly-Asp domain) embedded in ECM proteins, integrins can cluster and rearrange cytoskeletal structures, leading to the assembly of focal adhesions and actin stress fibers.[4] Focal adhesions serve as communication bridges, activating numerous signal transduction pathways and mediating the crosstalk between the actin cytoskeleton and ECM.[5]

Since focal adhesions play a central role in determining cell behaviors, it is interesting to fabricate extracellular-mimetic substrates that can be used to probe and better understand the influence of environmental cues on the formation of integrin clusters. To investigate the effect of ligand affinity on the number and size of focal adhesions, Kato and Mrksich immobilized a low-affinity, linear RGD ligand and a high-affinity, cyclic RGD ligand at identical densities on self-assembled monolayers

of alkanethiolates. 3T3 Swiss fibroblasts deposited on the surface modified with the cyclic RGD ligand developed twice the number of focal adhesions compared to the linear RGD ligand, while the average adhesion size was smaller.[6] In addition to composition, another study showed that the physical state of the ECM could regulate the differential assembly of adhesion sites. More specifically, it was demonstrated that physisorbed fibronectin could be reorganized into fibrils by attached cells. This behavior was mainly associated with $\alpha_5\beta_1$ integrin and led to the formation of elongated fibrillar adhesion structures. In contrast, covalently immobilized fibronectin complexed predominantly with $\alpha_v\beta_3$ integrin and the cells formed classic focal adhesions.[7] These observations have stimulated the design and synthesis of polymer materials with controlled physicochemical surface characteristics for regulating the proliferation and differentiation of endothelial cells into vascular-like structures.[8]

An appealing goal for material scientists is to develop instructive materials with fine-tuned physical properties and biochemical functionalities that could provide precise extracellular cues for regulating cellular behaviors and functions.[9,10] In this context, protein engineering provides a promising approach that allows extraordinary control over material properties and cellular responses.[11] Previously, an artificial polypeptide scaffold for protein immobilization onto solid substrates via heterodimeric coiled-coil association was developed.[12] Since it is straightforward to engineer leucine zippers with various affinities,[13,14] this technique permits diversity in the association strength of proteins linked to a surface. A step further is to generate

protein walkers that have different lateral mobility rates on a surface, which, if presenting cell adhesion ligands, may influence the clustering of integrin receptors.

4.2 Materials and methods

4.2.1 Cloning strategy

Recombinant genes encoding leucine zipper variants (Table 4.1) were created through polymerase chain reaction (PCR), assembling a mixture of multiple overlapping synthetic oligonucleotides designed by DNAWorks software. As indicated in Figure 4.1, proteins containing dimeric or trimeric leucine zipper variants were cloned. For the zipper domain ZE, for example, a previously constructed plasmid (pQE60_FNZE-FN-ZE) was digested with RsrII to obtain the $FNC_{10}ZE$ fragment. This fragment was multimerized by ligating monomers with T4 DNA ligase. Both the monomer and the dimer were ligated into pQE60_ZE to yield pQE60_ZE-FN-ZE and pQE60_ZE-FN-ZE-FN-ZE. All the resulting plasmids were transformed into the BL21 strain of *E. coli* by heat shock techniques.

Table 4.1 **Amino acid sequences of synthesized leucine zipper variants.**

Peptide	*def gabcdef gabcdef gabcdef gabcdef gabcdef gabcd*
ZR	LEI RAAALRR RNTALRT RVAELRQ RVQRLRN EVSQYET RYGPL
RE_3R	LEI RAAALRR ENTALET EVAELEQ EVQRLEN RVSQYRT RYGPL
RE_2RE	LEI RAAALRR ENTALET EVAELEQ RVQRLRN EVSQYET RYGPL
ZE	LEI EAAALEQ ENTALET EVAELEQ EVQRLEN RVSQYRT RYGPL

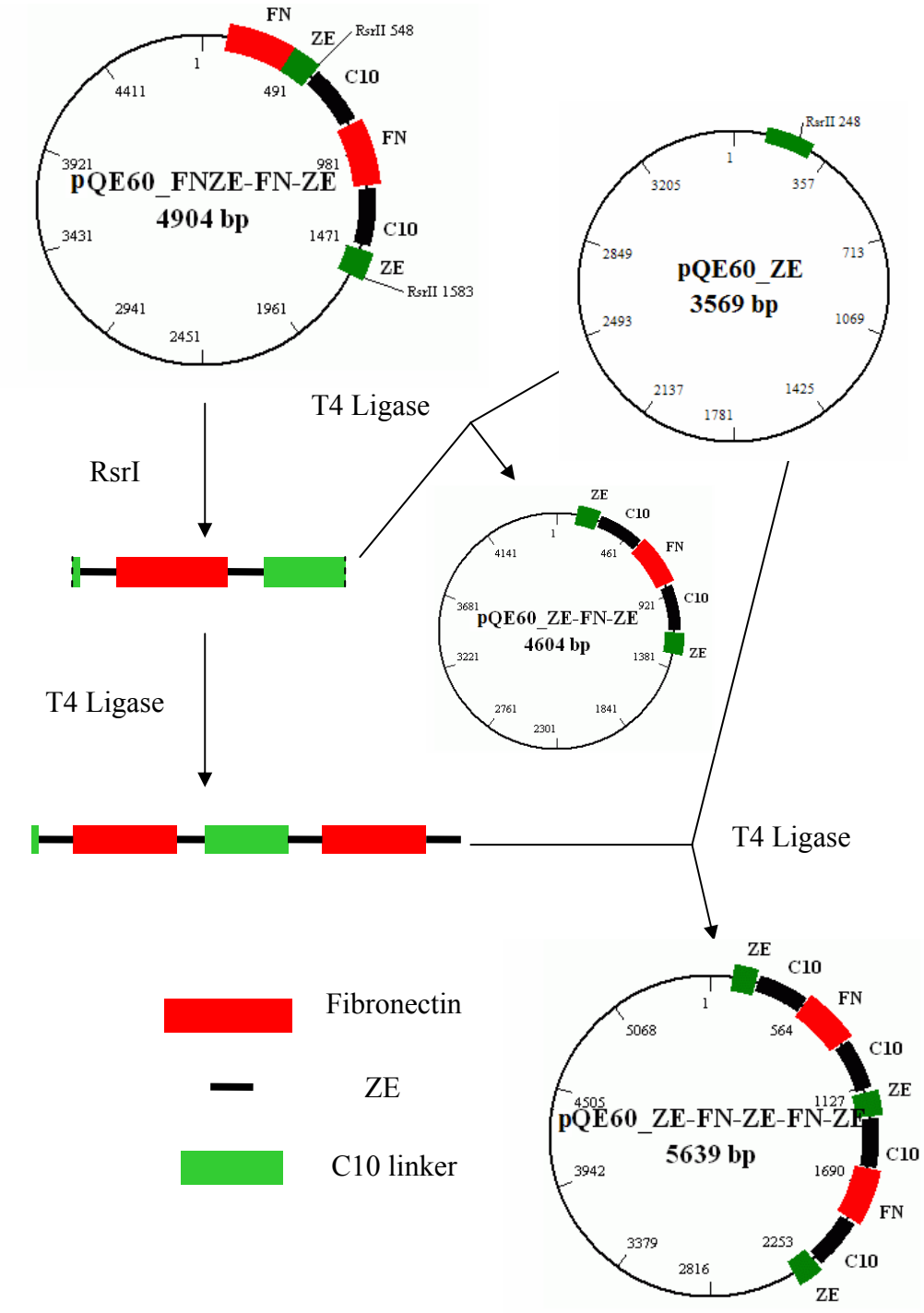


Figure 4.1 Cloning strategy for proteins containing multimeric leucine zipper variants.

4.2.2 Expression and purification of proteins

Transformed *E. coli* cells were grown in one liter 2XYT medium containing ampicillin and chloramphenicol at 37°C to an OD_{600} of 1.0. Overnight expression was induced upon the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). All the proteins were purified using Ni-NTA columns, and sample purity was assessed by SDS-PAGE and MALDI-TOF mass spectrometry.

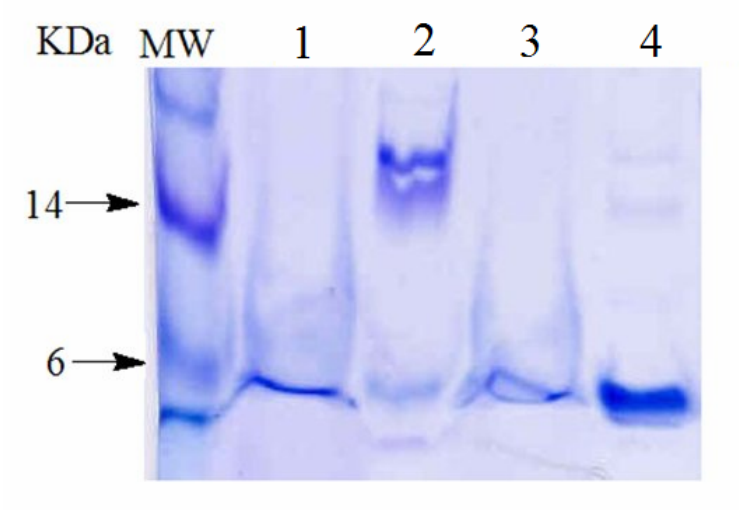


Figure 4.2 SDS-PAGE analysis of purified RE_3R and RE_2RE peptides (both contain His-tag). Proteins were co-expressed with ZRELF (without His-tag) to avoid *in vivo* degradation. Lane 1: RE_3R, Lane 2: a mixture of RE_3R and ZRELF was applied to the Ni-NTA agarose affinity column, washed with urea (pH=7.0) and eluted with urea (pH=4.5), Lane 3: RE_2RE, Lane 4: a mixture of RE_2RE and ZRELF was applied to the Ni-NTA agarose affinity column, washed with urea (pH=7.0) and eluted with urea (pH=4.5). Note that even after urea wash, ZRELF still associated with RE_3R in the column. The relative intensities of ZRELF bands (17 kDa) suggest that RE_3R binds more strongly than RE_2RE with ZR.

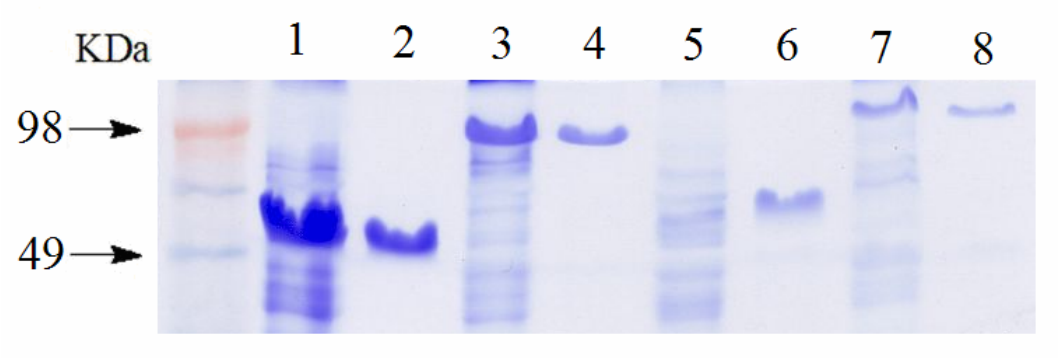


Figure 4.3 SDS-PAGE analysis showing expression and purification of proteins containing multimeric leucine zipper variants. Lane 1:cell lysate (RE_2RE dimer), Lane 2: Purified RE_2RE dimer, Lane 3: cell lysate(RE_2RE trimer), Lane 4: Purified RE_2RE trimer, Lane 5: cell lysate (ZE dimer), Lane 6: Purified ZE dimer, Lane 7: cell lysate(ZE trimer), Lane 8: Purified ZE trimer.

4.2.3 Circular dichroism (CD) spectrometry

CD spectra were obtained using a Model 62A DS Aviv Circular Dichroism Spectrometer (Lakewood, NJ). All samples were dissolved in 12.5 mM potassium phosphate (pH 7.4), 150 mM KCI, 1 mM EDTA; a 300 μL aliquot of these samples was used in the experiments. Homodimeric proteins (ZE, RE_2RE, RE_3R, and ZR) were prepared at a concentration of 20 μM. In addition, 20 μM ZE, RE_2RE, and RE_3R were individually mixed with 20 μM ZR to form heterodimers at a final concentration of 10 μM. Wavelength scans were obtained with the monochromator set to scan from 300 nm to 190 nm. Temperature scans were performed by scanning continuously from 10 °C to 90 °C at 222 nm in 2 °C increments.

4.2.4 Fluorescence recovery after photobleaching (FRAP)

To explore fluorescence recovery after photobleaching, proteins of interest were conjugated to Cy3 NHS ester supplied by Amersham Biosciences (Piscataway, NJ). The dye-labeled proteins were separated from excess dyes by dialysis against water for several days. The fluorescent proteins were immobilized onto a ZR-ELF functionalized surface as described previously and a DPSS 532-75 diode was used to excite the Cy3 fluorophore. Photobleaching was accomplished upon irradiation with 100% laser power for 2 minutes. Subsequent fluorescent recovery was monitored using the Achroplan 20x/0.5 W objectives of the Carl Zeiss LSM 5 Live microscope (Thornwood, NY).

4.3 Results and discussion

4.3.1 Design and characterization of heterodimeric leucine zipper pairs

The ZE/ZR heterodimer leucine zipper pair was derived from the vitellogenin-binding protein (VBP), a bZIP homodimer.[15] 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.[16] Thus, both ZE and ZR homodimers have four pairs of repulsive electrostatic interactions, whereas the ZE/ZR heterodimer contains four pairs of attractive salt bridges. To tune the heterodimerization affinities of the leucine zipper pairs, we also synthesized two modified acidic peptides, RE_3R and RE_2RE (Table 4.1), which form a decreasing

number of attractive salt bridges with ZR. The conformations of the synthetic peptides in solution were studied by CD (Figure 4.4). All three heterodimers exhibited an α-helix-like CD spectrum with large molar ellipticity minima values at 222 and 208 nm. The greatest negative ellipticity of the ZE/ZR heterodimer suggests the strongest helix-helix interactions. In addition, the stability of the coiled coils was determined by thermal denaturation experiments, where the changes in ellipticity at 222 nm were monitored (Figure 4.5). The data indicated a melting point of 74°C for RE_2RE/ZR. Since the other complexes were not completely melted at 90°C, their melting points could not be determined. However, the CD spectrum still showed that ZE/ZR pair was more stable than RE_3R/ZR pair, which in turn was more stable than the RE_2RE/ZR pair.

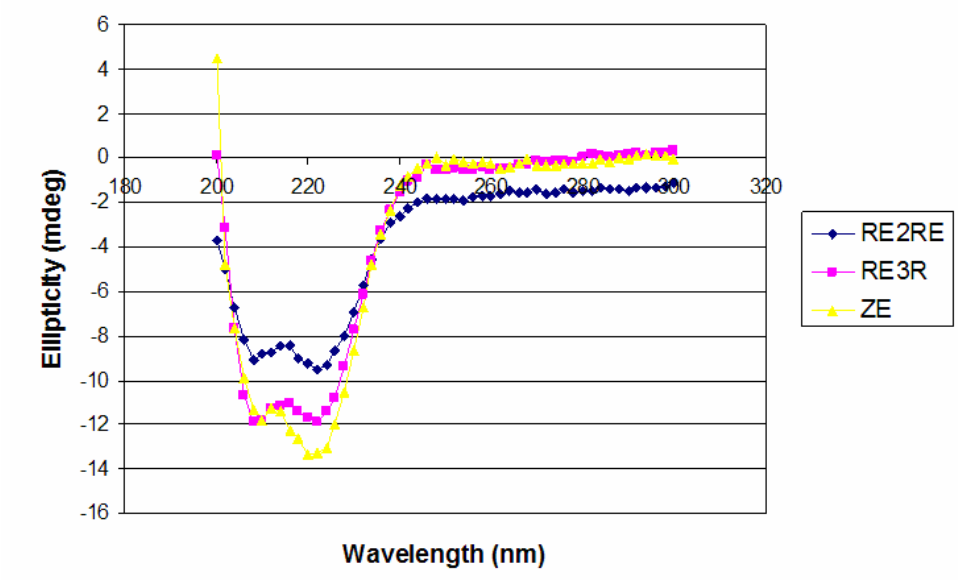


Figure 4.4 CD spectral data of RE_2RE, RE_3R, and ZE complexes to the basic ZR. All three heterodimers exhibited an α-helix-like CD spectrum with large molar ellipticity minima values at 222 and 208 nm. The greater negative ellipticity suggests stronger helix-helix interactions.

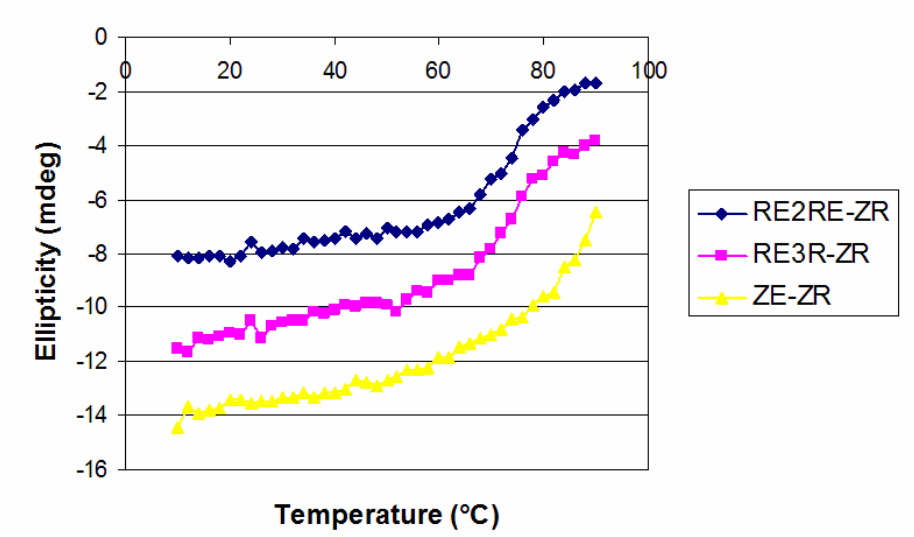


Figure 4.5 Thermal denaturation curves of RE_2RE, RE_3R, and ZE complexes to ZR. Temperature scans were performed by scanning continuously from 10°C to 90°C at 222 nm in 2 °C increments. The data suggested a melting point of 74°C for RE_2RE/ZR. The other complexes were not completely melted at 90°C, so melting points could not be determined. However, the spectrum showed that ZE/ZR was more stable than RE_3R/ZR, which in turn was more stable that RE_2RE/ZR.

4.3.2 Synthesis of protein walkers and measurement of lateral mobility

Utilizing the newly created leucine zipper pair variants, protein walkers were constructed to contain multiple sites for binding to ZRELF functionalized surfaces. Figure 4.6 illustrates a hypothetical model for lateral mobility using artificial protein walkers. In this scheme, one strand of the protein walker dissociates from the surface and then reattaches to the surface at a new binding site. The other strands may remain fixed or participate in strand exchange, allowing the protein walkers to move

laterally across the surface. The attachment of the protein walkers to the surface makes this movement stepwise, preventing diffusion of protein walkers into the solution.

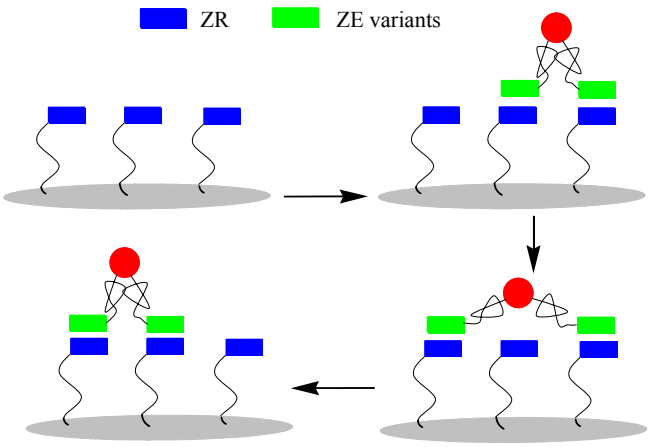
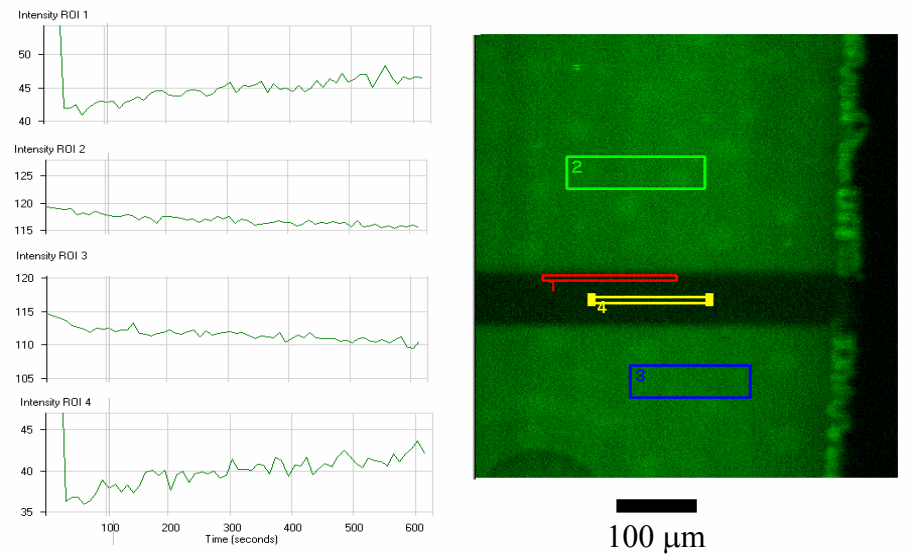


Figure 4.6 A hypothetical model: proteins containing multimeric binding motifs "walk" across the surface through strand exchange.

Based on the hypothetical model, we synthesized four proteins containing either two or three binding sites (either ZE or RE_2RE). These proteins were labeled with the fluorescent dye Cy3. After immobilization of these proteins on ZRELF functionalized glass substrates, the fluorescence intensity of four regions was monitored continuously over a period of ten minutes after photobleaching (Figure 4.7). The non-photobleached regions {ROI (region of interest) 2 & ROI 3} and the center of the photobleached region (ROI 4) were monitored as controls. Both ROI 2 and ROI 3 regions showed a steady decrease in intensity over time, suggesting a gradual dissociation of immobilized proteins from the surface. The ROI 4 region showed a

(a)



(b)

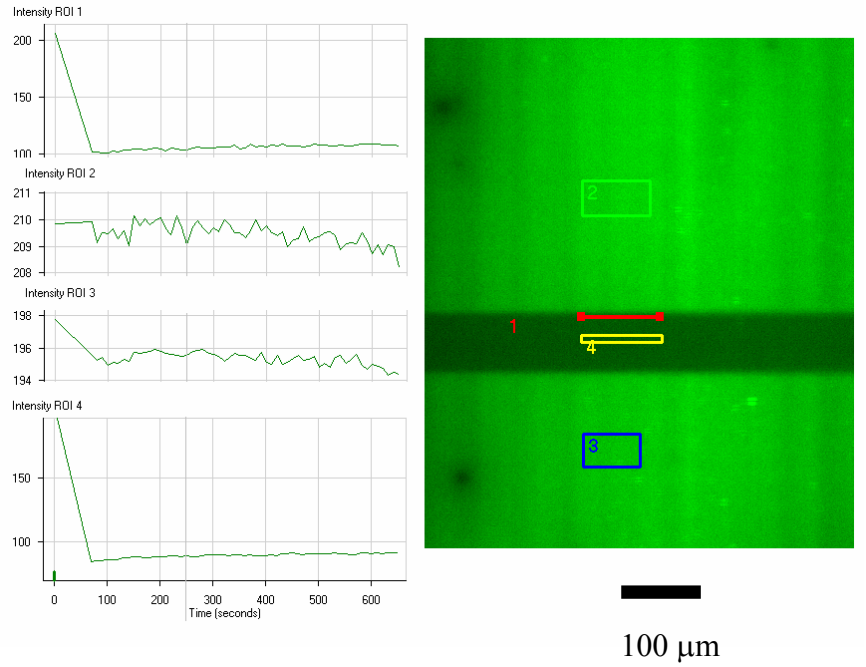


Figure 4.7 FRAP monitoring of protein walkers. (a) RE_2RE-FN-RE_2RE, (b) RE_2RE-FN-RE_2RE-FN-RE_2RE. The non-photobleached region (ROI 2 & ROI 3) and the center of the photobleached region (ROI 4) were monitored as controls. Both ROI 2 and ROI 3 regions showed a steady decrease in intensity over time. The ROI 4 region showed a steady increase in intensity over time. The fluorescence intensity of the interface between the photobleached region and the non-photobleached region (ROI 1) recovered at a similar level, indicating that self-recovery of photobleached dyes was the dominant factor in fluorescence increase rather than cross-diffusion of proteins between the photobleached region and the non-photobleached region.

steady increase in intensity over time, which suggested gradual self-recovery of the photobleached Cy3 fluorophores. However, the fluorescence intensity of the interface between the photobleached region and the non-photobleached region (ROI 1) recovered at a similar level, indicating that self-recovery of photobleached dyes was the dominant factor in the fluorescence increase rather than diffusion of proteins between the photobleached and non-photobleached regions. Thus, FRAP measurements did not provide evidence of lateral mobility of either dimeric or trimeric proteins.

Several possibilities may account for the lack of evidence for lateral mobility. First, in order to achieve efficient movement, leucine zipper pairs should have extremely fast association-dissociation kinetics so that the legs of protein walkers could effectively probe the surface without total detachment of the entire protein from the surface. Second, the expected lateral mobility might occur on a smaller scale than could be observed with the LSM 5 Live microscope (approximately 1 μm resolution). High resolution experimental setups such as single molecule spectroscopy may be required to observe the movement of such protein walkers. Finally, it is possible that lateral mobility was not occurring due to surface saturation. For example, when a leucine zipper became detached, it might reattach to the same ZR site and there was no net movement.

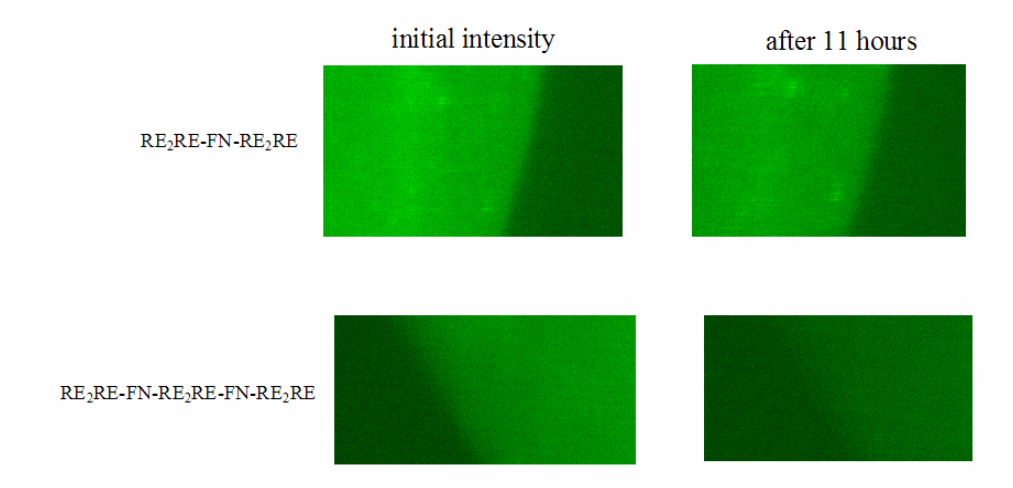


Figure 4.8 Spotting proteins on ZRELF surface and monitoring fluorescence change in PBS buffer after 11 hours.

To address this concern, an additional experiment was performed to examine the potential saturation effect. Both RE_2RE-FN-RE_2RE and RE_2RE-FN-RE_2RE-FN-RE_2RE were spotted onto a ZRELF functionalized surface. The fluorescence of immobilized protein spots was monitored over a period of eleven hours (Figure 4.8). The highly fluorescent regions were saturated with proteins, while dark regions were devoid of surface-bound ligands. Therefore, if proteins could move from the saturated regions to empty regions, the edge should become more fluorescent over time. This result was not observed; instead, there was a sustained dissociation of proteins from the surface into the medium as indicated by the decrease of fluorescence. Thus, the saturation effect does not account for the lack of lateral mobility of the protein walkers.

4.4 Conclusion

We have designed and synthesized several variants of leucine zipper pair that have tunable heterodimerization affinities. Four proteins containing either two or three of these binding motifs were created. However, we did not observe lateral mobility of either dimeric or trimeric proteins.

4.5 References

(1) Alberts, B.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K.; Walter, P. *Molecular Biology of the Cell. 4th ed.* New York: Garland Science, 2002.

(2) Boudreau, N.; Bissell, M. J. *Curr. Opin. Cell Biol.* **1998**, *10*, 640.

(3) Buckley, C. D.; Rainger, G. E.; Bradfield, P. F.; Nash, G. B.; Simmons, D. L. *Mol. Membr. Biol.* **1998**, *15*, 167.

(4) Burridge, K.; Chrzanowska-Wodnicka, M. *Annu. Rev. Cell Dev. Biol.* **1996**, *12*, 463.

(5) Romer, L. H.; Birukov, K. G.; Garcia, J. G. *Circ. Res.* **2006**, *98*, 606.

(6) Kato, M.; Mrksich, M. *Biochemistry* **2004**, *43*, 2699.

(7) Katz, B. Z.; Zamir, E.; Bershadsky, A.; Kam, Z.; Yamada, K. M.; Geiger, B. *Mol. Biol. Cell* **2000**, *11*, 1047.

(8) Pompe, T.; Markowski, M.; Werner, C. *Tissue Eng.* **2004**, *10*, 841.

(9) Langer, R.; Tirrell, D. A. *Nature* **2004**, *428*, 487.

(10) Lutolf, M. P.; Hubbell, J. A. *Nat. Biotechnol.* **2005**, *23*, 47.

(11) Maskarinec, S. A.; Tirrell, D. A. *Curr. Opin. Biotechnol.* **2005**, *16*, 422.

(12) Zhang, K. C.; Diehl, M. R.; Tirrell, D. A. *J. Am. Chem. Soc.* **2005**, *127*, 10136.

(13) Krylov, D.; Mikhailenko, I.; Vinson, C. *EMBO J.* **1994**, *13*, 2849.

(14) Mason, J. M.; Arndt, K. M. *ChemBioChem.* **2004**, *5*, 170.

(15) Krylov, D.; Barchi, J.; Vinson, C. *J. Mol. Biol.* **1998**, *279*, 959.

(16) Moll, J. R.; Ruvinov, S. B.; Pastan, I.; Vinson, C. *Protein Sci.* **2001**, *10*, 649.