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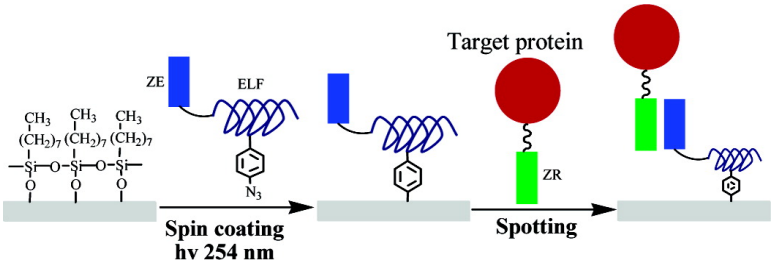

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Artificial Polypeptide Scaffold for Protein Immobilization

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Protein microarray technologies are beginning to advance the field of proteomics by providing miniaturized platforms to probe the interactions and functions of proteins.[1] Presenting proteins in dense arrays enables the rapid screening of thousands of molecular events in a single experiment. This capability should facilitate the elucidation of protein profiles in organisms, the discovery of novel protein functions, and the development of systems-level understanding of biological phenomena.[2] The utility of microarrays to probe protein−protein interactions has been demonstrated by Zhu et al., where new calmodulin- and phospholipid-binding proteins were identified by screening a full scale yeast proteome microarray.[3] More recently, Nielsen et al. used antibody microarrays to profile the activation of receptor tyrosine kinases and analyze signal transduction networks in mammalian cells.[4]

Despite the growing success of protein microarrays, it remains a central challenge to develop simple and general techniques to immobilize functional proteins onto solid supports. In certain cases, conventional immobilization methods (such as physical adsorption or covalent binding through lysine and cysteine residues) render active sites inaccessible or even denature proteins.[5] This difficulty may be overcome by engineering site-specific attachment to a substrate through expression of recombinant fusion proteins bearing affinity tags. For example, the immobilization of his tag fusion proteins onto Ni−NTA-functionalized slides has been shown to maintain higher protein activity than direct attachment to aldehyde slides.[3] Nevertheless, his tags do not necessarily provide a general approach to array fabrication because the binding interaction is sensitive to pH and to some common buffer components.[6] To solve this problem, a variety of alternative strategies are being developed. One such approach uses the strong interaction between avidin and biotin to immobilize proteins in combination with in vivo or in vitro biotinylation.[7] Other methods introduce polypeptide tags to effect selective and covalent attachment.[8] Optimal immobilization schemes should be characterized by simple cloning schemes, efficient protein expression, selective affinity, and simple surface chemistry. This formidable challenge requires the design of new biomaterials that maintain protein architecture and allow specific chemistries to be utilized for immobilization.[9]

We have approached this problem by creating an artificial polypeptide scaffold **1** that can be used to immobilize recombinant proteins on substrates (Figure 1). The polypeptide contains separate surface anchor and protein capture domains and uses an artificial amino acid to covalently crosslink the polypeptide to surfaces. The protein capture domain functions through coiled coil association of a designed parallel heterodimeric leucine zipper pair, designated ZE and ZR. These structures are based on the sequences developed by Vinson et al.[10] with minor modifications (see Supporting Information). Vinson et al. showed that this leucine zipper system has a heterodimerization affinity of 10^{-15} M, while homodimerization affinities are in the micromolar range. The acidic component ZE is incorporated into **1** as the protein capture domain, and the basic portion ZR is fused to target proteins as an affinity tag. An

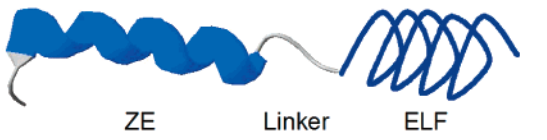
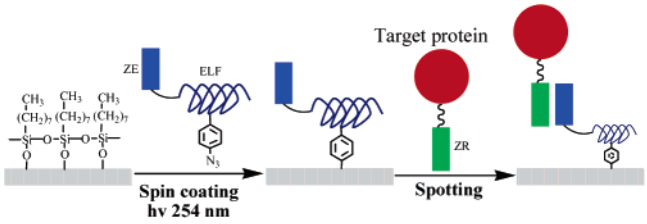


Figure 1. Design of the artificial polypeptide scaffold **1** and related amino acid sequence.

Scheme 1. Surface Functionalization and Coiled Coil Mediated Immobilization of Proteins



important part of the scaffold design is introduction of an elastin mimetic domain ELF for surface anchorage. ELF consists of five repeats of 25 amino acids with the sequence $(VPGVG)_2VPG\boldsymbol{F}G(VPGVG)_2$. Because of its hydrophobic character, ELF provides strong adhesion to hydrophobic surfaces.[11] Moreover, because **1** is expressed in a bacterial host harboring a mutant *E. coli* phenylalanyl−tRNA synthetase (A294G), the phenylalanine residues in the ELF domain are partially replaced by a photoreactive nonnatural amino acid, *para*-azidophenylalanine.[12] This moiety can be used to generate covalent linkages to substrates upon UV irradiation. To reduce possible steric hindrance, the ZE and ELF domains are linked by a flexible spacer of 14 amino acids. The designed protein sequence was reverse-translated based on the codons most often used in *E. coli* and expressed in the phenylalanine auxotroph strain AF−IQ.[13] Typical yields were 50 mg/L, and the rate of incorporation of *para*-azidophenylalanine was approximately 45% as determined by amino acid analysis.

The successful design, in vivo expression, and purification of **1** allowed us to prepare a functionalized surface for protein immobilization (Scheme 1). In this procedure, a solution of **1** (0.8 mg/mL in 50% trifluoroethanol) was spin-coated on glass slides that were pretreated with octyltrichlorosilane (OTS) to make them hydrophobic. Once dry, the protein films were irradiated with UV light.[14] Irradiation of the films covalently crosslinked the protein to the substrate through photodecomposition of the aryl azide groups[15] of *para*-azidophenylalanine. Any noncovalently bound protein was removed by sonicating in 80% DMSO for 20 min. Measurements of the water static contact angle indicated marked changes in wettability upon formation of the protein film; the contact angle was 60° after photocrosslinking and sonication as compared

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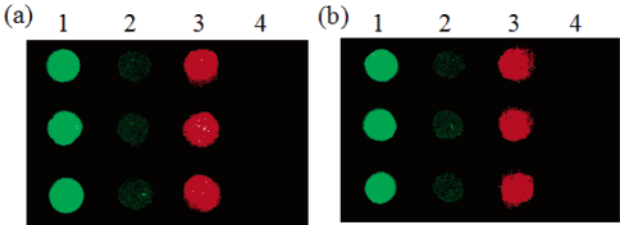

Figure 2. Immobilization and detection of proteins on polypeptide-functionalized surfaces. Printed spots were detected with a mixture of cy3-anti-GST and alexa647-anti-GFP: (a) 5 μM purified proteins (1) GST-ZR, (2) GST, (3) GFP-ZR, (4) GFP; and (b) cell lysates (1) GST-ZR, (2) GST, (3) GFP-ZR, (4) GFP. The spots are 200 μm in diameter.

to 107° for the initial OTS substrates. In a final step, films were blocked with 1% casein solution to reduce nonspecific protein adsorption.

Green fluorescent protein (GFP) and glutathione-*S*-transferase (GST) were chosen as model systems to test the efficacy of the functionalized surface for protein immobilization. These proteins were expressed in vivo with the ZR tag fused to their C-termini. Proteins lacking the fusion tag were expressed as controls. Purified proteins at a concentration of 5 μM were spotted onto the surface to generate protein microarrays (Figure 2a). The arrays were incubated in a humid chamber for 1 h and then thoroughly washed twice with PBS−Tween buffer (PBS plus 0.5% Tween-20) to remove nonspecifically bound protein. Each array was probed with a mixture of cy3-anti-GST and alexa647-anti-GFP (4 μg/mL each), washed, and scanned with a Genepix microarray scanner. As expected, spots containing fusion proteins showed much stronger signals than control proteins. The average signal-to-noise ratio, SNR,[16] of GST-ZR was 196 ± 20 and that of GFP-ZR was 43 ± 4. Without the ZR fusion, GST spots yielded weaker detectable signals (SNR = 15 ± 3[17]), and GFP spots could not be distinguished from background. The sensitivity of the method is high; SNR ratios of 4 or 2 (GST-ZR or GFP-ZR) were obtained when proteins were spotted at concentrations as low as 50 nM. These qualities encouraged us to examine immobilization of ZR-tagged proteins directly from crude cell lysates. Cell lysates containing over-expressed fusion or control proteins were spotted onto functionalized surface and detected by the procedure described above. As shown in Figure 2b, significant protein attachment occurred only when the complementary zipper fusion tag was present.

The protein immobilization method presented here has several advantages over traditional methods. First, spin coating plus photoimmobilization provides a simple and convenient route to uniform protein films. This procedure requires a fabrication time of minutes and yields dense surface coverage. Second, the heterodimeric association of this leucine zipper system is highly specific and stable. In fact, we have found that the heterodimer forms even in 8 M urea solution over a wide range of pH values (tested from pH 4.0 to 8.0). This stability expands the range of working conditions to stringent situations where other methods are not applicable. Third, considering the relatively small size of the zipper tag (43 amino acids), it is unlikely that the function of the fusion proteins will be compromised. Finally, direct immobilization of fusion proteins from crude cell lysates makes it feasible to fabricate protein arrays in high throughput fashion by eliminating time-consuming and costly purification steps.

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Supporting Information Available: Detailed procedures of cloning, protein expression, purification, and microarray related experiments (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) SNR = (signal background)/(standard deviation of background).
(17) The nonspecific adsorption signal was caused by rapid drying of microspots during printing. We did not observe signals from GST spots if we used a manual pipet to spot 1 μL of protein solutions.

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