Elastin−Calmodulin Scaffold for Protein Microarray Fabrication

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In this work, we report a new method to reversibly immobilize proteins to a surface in a functionally active orientation directly from cell lysate by employing a fusion protein consisting of a thermal-responsive elastin (ELP) domain as the surface anchor and a calcium-responsive calmodulin (CalM) domain for protein capturing. Incorporation of an M13 tag into recombinant proteins enables not only easy surface immobilization but also direct purification from cell lysates. The feasibility of concept was demonstrated using the M13-tagged yellow fluorescent protein (M13-YFP). The ELP−CalM functionalized surfaces were shown to capture M13-YFP directly from cell lysate through the specific calmodulin-M13 association in a calcium-dependent manner. We also demonstrated that immobilization is reversible; the bound proteins were released from the surface in the presence of EDTA.

Introduction

Protein microarrays have become an important tool for advancing disease diagnostics and proteomics analysis due to their ability to provide high-throughput screening.[1,2] Despite this promising capability, immobilization of proteins in a spatially defined pattern remains a major hurdle for the widespread usage of protein microarrays. Conventionally, both physical adsorption and covalent coupling to an array surface have been used. Unfortunately, concerns with active site accessibility and protein denaturation render these methods undesirable. Noncovalent immobilization has also been made possible by the use of recombinant fusion proteins carrying a specific affinity tag such as polyhistidine, carbohydrate-binding proteins, and glutathione S-transferase.[3] However, the need of prior protein purification and chemical modification of the surface before array fabrication are the major disadvantages. An optimal immobilization approach would offer the ability to fabricate a protein array directly from the cell lysate with high affinity and expression without complex purification and surface chemistry.

Calmodulin (CalM) is a calcium-binding protein which has been demonstrated to work as an affinity ligand[4] for the purification of recombinant proteins containing the CalM-binding M13 peptide.[5] More importantly, the M13 peptide, a 26 AA residue part of the myosin light-chain kinase, binds to CalM with nanomolar affinity in the presence of $CaCl_2$[6] and has been shown to have no effect on the partner protein activity due to its small size (4 kDa).[7] The CalM−M13 system offers high specificity, as no endogenous proteins from microorganisms could interact with CalM, making it ideal for protein immobilization directly from cell lysates.

We report here a new approach for fabricating protein arrays where a surface-oriented elastin−calmodulin (ELP−CalM) scaffold is used to capture recombinant proteins containing an M13 tag onto the surface (Figure 1). Surface anchoring of the ELP−CalM scaffold is accomplished via temperature-triggered hydrophobic interaction between the ELP domain and the hydrophobic surface.[8] Moreover, the presence of the ELP domain enables simple protein purification by the inverse phase transition.[9] Immobilization of M13-tagged proteins occurs through Ca^{2+}-mediated association between CalM and the M13 tag. This protein-based approach provides a simple and robust platform for site-specific protein immobilization in a functionally active orientation without complex covalent modification.

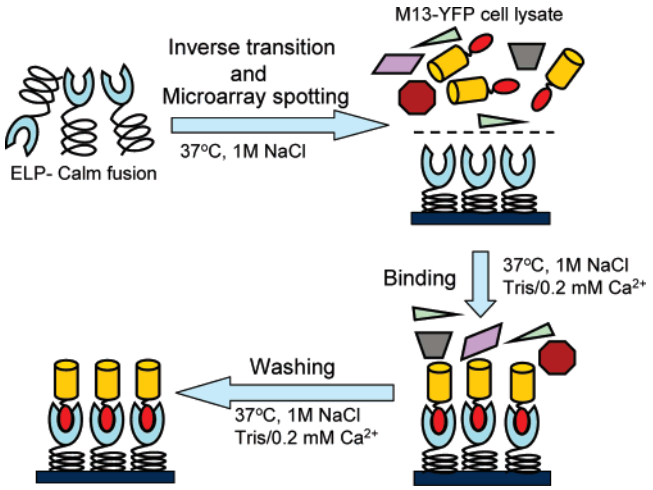

Figure 1. Schematic procedure to capture M13-tagged proteins directly from cell lysate. ELP−CalM fusions are spotted onto the glass slide by taking advantage of the temperature-triggered hydrophobic interaction.

Experimental Section

Production of ELP−CalM and M13−YFP. ELP−CalM was designed to contain a CalM domain linked to the C-terminal of the elastin domain with 145 repeats (Figure 1). In parallel, an M13−YFP fusion was created by adding the 26 amino acids M13 tag to the N-terminal of YFP. The calmodulin gene and the M13−YFP gene fragment were PCR amplified using vector YC2.3[10] as the

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template. Expression of both proteins was achieved in *E. coli* BLR(DE3) using the pET vector system. Purification of ELP−CalM was achieved by two cycles of inverse temperature transition as described before.[9] Purity of the protein was determined by SDS-PAGE electrophoresis followed by Coomassie blue staining.

Purification of M13-YFP. Cell extract containing M13−YFP was mixed with 5 nmol of ELP−CalM in the presence of 0.2 mM $CaCl_2$ and 1 M NaCl in 10 mM Tris buffer (pH 7.0). The solution was gently mixed by rotating at room temperature for 30 min. The ELP−CalM−M13−YFP complex was recovered by incubation at 37 °C for 5 min and centrifuged at the same temperature for 5 min at 14 000 *g*. The resulting pellet was resuspended in 100 μL of ice-cold 10 mM Tris buffer (pH 7.0)/0.2 mM $CaCl_2$. The same cycle was repeated one additional time. The recovery of M13−YFP was determined by fluorescence measurement using a 96 well microplate reader (POLARstar Optima, BGM Labtechnologies, Inc.) with excitation at 485 nm and emission at 520 nm.

Protein Array Fabrication. Glass slides were coated with a self-assembled monolayer (SAM) formed from octadecyltrichlorosilane (OTS, $CH_3(CH_2)_{17}SiCl_3$) as described before.[11] ELP−CalM−M13−YFP complex was prepared as described above. To trigger the hydrophobic aggregation, either ELP−CalM or ELP−CalM−M13−YFP was incubated at 37 °C in the presence of 1 M NaCl. The aggregated complex was then loaded into a 96 well plate and spotted on the OTS-coated glass slide using a Virtek DNA microarrayer (Bio-Rad) with Stealth Micro Spotting pins (Telechem International, Inc., CA). After spotting, the glass slides were immersed in a bovine serum albumin (BSA) solution (20 mg/mL in Tris buffer) for 2 h and then rinsed with warm Tris buffer (37 °C). For direct immobilization from cell lysate, ELP−CalM was first spotted onto the glass surface as described. The modified surface was then incubated with 20 μL of M13−YFP cell lysate for 20 min. The glass slides were rinsed with washing buffer for 5 min. Control experiments were performed with spotted elastin control protein ELP78 without the calmodulin moiety to address possible nonspecific binding interactions. Fluorescence scans were acquired by a ScanArray Express microarray scanner (Packard Biosciences).

Results and Discussion

An ELP domain containing 145 VPGVG repeats was used to fuse with calmodulin. This particular ELP was chosen to enable a mild phase transition condition. A flexible linker was added to minimize the interference and steric hindrance. Production of the ELP−CalM fusion was achieved in *E. coli* BLR(DE3) using the pET expression system. ELP−CalM was purified by two cycles of temperature-triggered precipitation and resolubilization (inverse temperature transition), demonstrating that the fusion protein retains the reversible hydrophobic−hydrophilic transition property of ELP. Purity of the fusion protein was verified by SDS-PAGE (data not shown). Typically, 250 mg/L of ELP−CalM was obtained.

The functionality of the CalM domain was demonstrated by the ability of ELP−CalM to purify the M13-tagged protein directly from the cell lysate. YFP was chosen as an example, since it can be easily detected by fluorescence measurement. Separation of M13−YFP from cell lysate was achieved by coprecipitation with ELP−CalM through Ca^{2+}-mediated interaction between the CalM domain and the M13 tag. The efficiency of purification was monitored by measuring the fluorescence intensity of YFP at different steps of the process. Water-soluble ELP−CalM was mixed with cell lysate containing M13−YFP in Tris buffer containing 200 μM $CaCl_2$ (Tris/Ca) at room temperature for 30 min, and the ELP−CalM−M13−YFP complex was separated from other cellular proteins by two cycles of precipitation with 1 M NaCl at 37 °C, centrifugation, and resolubilization in 4 °C Tris/Ca buffer. More than 80% of the total fluorescence from the cell lysate was recovered with the final pellet (Figure 2), and the presence of M13−YFP was confirmed by SDS-PAGE (data not shown).

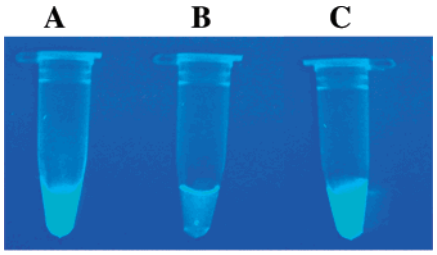

Figure 2. Purification of M13−YFP by ELP−CalM fusion. The fluorescent intensity in (A) the total cell lysate, (B) supernatant, and (C) resolubilized pellet after centrifugation.

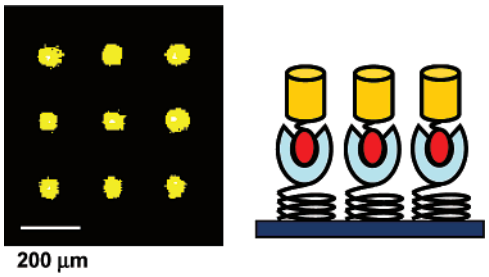

Figure 3. Fluorescence image of microarray fabricated by immobilization of the ELP−CalM/M13−YFP complex onto the hydrophobic surface of the OTS-coated glass slide.

Previously, we demonstrated that ELP−ProA-, ProG- or ProL-antibody complexes can be spotted onto a hydrophobic glass slide via temperature-triggered hydrophobic interaction between the ELP domain and the hydrophobic surface.[10] In a similar manner, any protein tagged with M13 bound to ELP−CalM can be spotted on a glass slide using the same hydrophobic interaction. To illustrate this, the glass slide was modified with a self-assembled monolayer of octadecyltrichlorosilane (OTS, $CH_3(CH_2)_{17}SiCl_3$) to achieve a water contact angle of 112°. The M13−YFP fusion was again employed due to the ease of direct fluorescent imaging. Formation and purification of the ELP−CalM−M13−YFP complex was achieved as described above. For immobilization, the ELP domain was triggered to its hydrophobic phase by the addition of 1 M NaCl to the complex solution at 37 °C. The complex was then loaded into a 96 well plate and spotted onto the glass slide using a DNA microarrayer. After washing three times with Tris-Cl buffer containing 0.2 mM $CaCl_2$, the glass slide was imaged with a fluorescent scanner. Figure 3 shows the fluorescence image of the immobilized protein array. Strong fluorescent signals were detected only on regions spotted with ELP−CalM−M13−YFP at a uniform size of about 80 μm and 200 μm in spacing. The interaction between CalM and M13 was very stable, as less than a 2% decrease in fluorescent intensity was observed even after ten more washes with the buffer.

To demonstrate the direct capturing of M13−YFP from cell lysate using the immobilized ELP−CalM, the ELP−CalM capturing scaffold was first immobilized onto a glass slide using the temperature-triggered hydrophobic interaction as described above. The glass surface was subsequently blocked with bovine serum albumin to reduce the nonspecific adsorption of other proteins to the surface. To test the efficacy of the functionalized glass surface for M13−YFP immobilization, cell lysate containing M13−YFP was incubated with the modified glass slide in the presence of 200 μM $CaCl_2$. After washing three times with Tris-

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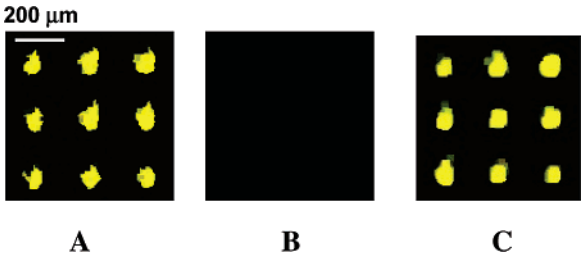

Figure 4. Fluorescence images of the ELP−CalM microarrays. (A) Binding of M13−YFP from the cell lysate on the ELP−CalM protein array matrix. (B) Stripping by EDTA. (C) Rebinding of M13−YFP on the same microarray.

Cl buffer containing 0.2 mM $CaCl_2$, the glass slide was imaged with a fluorescent scanner. Only spots preimmobilized with ELP−CalM lit up as expected, and a similar resolution of 80 μm in diameter and 200 μm in spacing was observed (Figure 4A). A control experiment using immobilized ELP protein with the CalM domain resulted in no observable fluorescence signals (data not shown), indicating that the calmodulin−M13 interaction was solely responsible for direct spotting of YFP from the cell lysate.

To verify that the immobilization of M13−YFP was indeed Ca^{2+} mediated, similar experiments were performed in the absence of Ca^{2+} with no distinct array observed (data not shown). Moreover, washing the immobilized array with 2 mM EDTA for 1 min completely removed all M13−YFP (Figure 4B), resulting in only background fluorescent intensity. Washing with EDTA has no effect on the surface-immobilized ELP−CalM scaffold, as an array with more than 80% of the original fluorescent intensity was observed when exposed again to cell lysate containing M13−YFP (Figure 4C).

In summary, we demonstrated a new concept for protein array fabrication where M13-tagged proteins are immobilized onto an array surface via Ca^{2+}-dependent binding to prepatterned ELP−CalM. The selective interaction between CalM and the M13 tag allows the use of cell lysate containing the target protein for direct immobilization without costly purification while maintaining the proteins under mild physiological conditions. The use of ELP−CalM as the capturing scaffold also enables fabrication without the need of complex chemical modification. More importantly, the present system is reversible, enabling the recovery of the bound protein−protein/ligand complex for subsequent analysis. This is particularly useful for the study of protein−protein and protein−small molecule interactions by proteome microarray analysis and may also be useful for lab-on-a-chip applications that require the capture and release of proteins directly from a complex mixture.

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