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Chemoselective Attachment of Biologically Active Proteins to Surfaces by Expressed Protein Ligation and Its Application for "Protein Chip" Fabrication

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Many experimental techniques in biology and biophysics, and applications in diagnosis and drug discovery, utilize proteins immobilized on solid substrates.[1] The use of arrays of proteins attached to solid supports is receiving increasing attention as a tool for exploring the function and potential relationships of all the proteins encoded in the genome.[1a] Protein arrays (or chips) are ideal reagents for such analysis in parallel fashion.[1d,e]

Most of the available methods for generating protein arrays rely on nonspecific adsorption of proteins or on the reaction of chemical groups within proteins with surfaces containing reactive groups.[2] In both cases, the protein is attached to the surface in random orientations. The use of recombinant affinity tags addresses the orientation issue.[3] However, in most cases the interactions of the tags are reversible and not stable over the course of subsequent assays or require large mediator proteins.[3c−f] The presence of such large protein mediators can potentially give rise to problems, especially in those applications where the attached proteins are needed to study protein/protein interactions with complex protein mixtures.[1a]

A direct covalent attachment between the protein and the surface using poly(ethylene glycol) (PEG) linkers eliminates the need for protein mediators. These linkers are well-known for their ability to prevent nonspecific interactions and also act as hydrophilic spacers minimizing any detrimental interaction between the attached protein and the solid surface.[1b] Control over the orientation of the attachment requires two unique and mutually reactive groups on the protein and the support surface. The reaction between these two groups should be highly chemoselective, thus behaving like a molecular "velcro".[4]

Here, we describe for the first time the use of "expressed protein ligation" (EPL)[5,8] for the creation of microscaled arrays of proteins covalently attached through their C-termini to a modified glass surface containing an N-terminal Cys poly(ethylene glycol) linker (Scheme 1). In this approach, the protein remains attached to the solid support through a stable peptide bond and is able to maintain its folded state and biological activity.

Key to our approach is the use of protein α-thioesters recombinantly generated using an engineered intein expression system.[6] The protein α-thioesters are covalently attached by "native chemical ligation"[7] through a glass surface modified with PEGylated thiol linkers **1** and **2**. This exquisitely specific reaction between polypeptide α-thioesters and N-terminal Cys-containing molecules has been extensively used for the synthesis, semisynthesis, and engineering as well as site-specific labeling of different proteins.[5,7,8] However, it has been never used before for the direct immobilization of proteins onto appropriate surfaces for creating protein microarrays.[12]

Scheme 1. Selective Immobilization of a Protein α-Thioester on a Cys-Containing Glass Surface

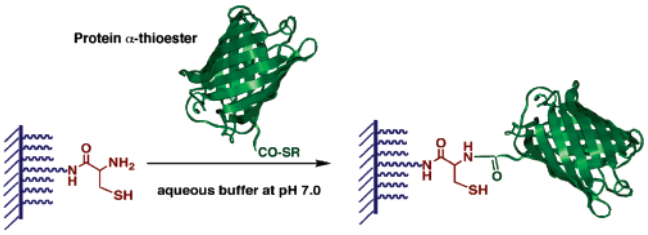
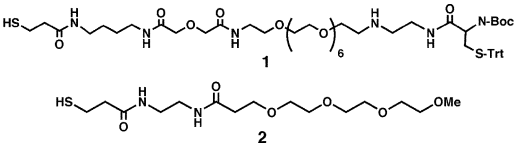


Chart 1. Chemical Structures of Linkers **1** and **2**



To test the suitability of EPL for this task, we used two fluorescent proteins, DsRed and EGFP (enhanced green fluorescent protein), as models. DsRed is a tetrameric red fluorescent protein and EGFP is a monomeric variant of the green fluorescent protein. In both cases, the proteins are fluorescent only if their tertiary and quaternary (DsRed protein shows red fluorescence only in the tetrameric state[9]) structures are kept intact. They served as controls to test if the native architecture of these proteins is altered during the attachment. Both protein α-thioesters were readily expressed in *E. coli* using an intein expression system.[5,6,8] To facilitate the site-specific attachment of the fluorescent protein α-thioesters onto a glass surface for the fabrication of protein microarrays, a glass slide was silanized with (3-acryloxypropyl)trimethoxysilane and then reacted with a mixture of PEGylated thiol linkers **1** and **2**, in a molar ratio of 1:5, respectively (Chart 1). Linker **1** contained a protected N-terminal Cys residue for the selective attachment of the α-thioester proteins, while linker **2** was used as a diluent to control the number of reactive sites on the surface. Linker **1** also contains a longer PEG moiety than linker **2** (Chart 1) to ensure that the reactive Cys groups were readily available to react with the corresponding protein α-thioester in solution. When the derivatization was complete, the protecting groups (N-Boc and S-Trt) of the Cys residue from linker **1** were removed by a brief treatment with trifluoroacetic acid. The surface was rinsed, neutralized, and quickly used for spotting (Figure 1a). As a control, a solution of EGFP with no α-thioester function was also spotted on the same slide. The ligation reaction was kept for 36 h in the dark at room temperature, and the protein-modified slide was then extensively washed. As shown in Figure 1a, only specific attachment between the α-thioester proteins and the Cys-containing glass surface was observed. No fluorescence signal was observed where the control EGFP protein lacking an α-thioester was spotted, thus indicating that this functionality is required for the chemoselective

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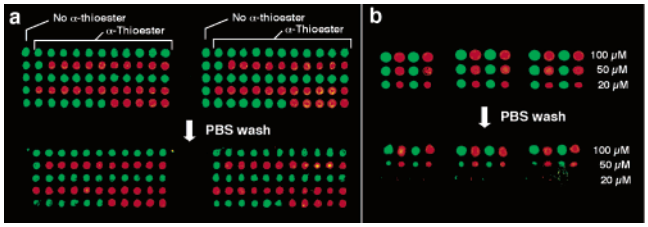

Figure 1. Selective attachment of EGFP (green) and DsRed (red) α-thioesters onto a Cys-containing glass slide. (a) Epifluorescence image of the glass slide after the protein spotting (top) and after PBS (phosphate buffer solution) washes (bottom). EGFP with no α-thioester was used as a control. Spotting was carried out using 100 μM protein solutions. (b) Effect of the protein concentration on the efficiency of attachment. Epifluorescence image after spotting (top) and PBS washes (botton).

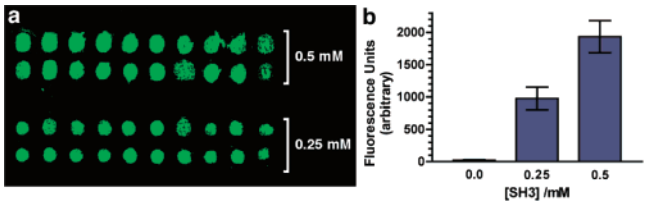

Figure 2. Selective attachment of the c-Crk N-terminal SH3 domain α-thioester onto a Cys-modified glass slide. (a) The SH3 domain was detected with an Alexa 488-labeled poly-Pro C3G-derived peptide. (b) Correlation between the fluorescence detected and the concentration of the spotted SH3 solution.

attachment. It is interesting to note that the immobilized DsRed protein retained its red fluorescence, thereby indicating that its tetrameric architecture was unaffected by the attachment to the PEGylated glass surface.

We also investigated the minimum protein concentration required for having selective attachment of the protein onto the Cys-containing glass surface. As shown in Figure 1b, different concentrations of EGFP and DsRed protein α-thioesters were spotted onto a Cys-containing glass slide and incubated for 36 h. As expected, the concentration of the protein was critical for an efficient attachment of the corresponding protein α-thioester to the derivatized glass slide. In both fluorescent proteins, the minimum concentration for acceptable levels of immobilization was found to be around 50 μM (Figure 1b).

Finally, we explored the ability to quantify protein/protein interactions. We used the N-terminal SH3 domain of the c-Crk protein adaptor as a model system for this purpose.[10] The SH3 protein domain α-thioester was expressed in *E. coli* as described above. Different concentrations of the SH3 protein domain α-thioester were spotted onto a Cys-containing glass slide as before (Figure 2a). After 36 h of incubation, the unbound protein was washed from the slide and the microarray was probed with an Alexa-488 labeled poly-Pro peptide derived from the C3G exchange factor protein.[10] The surface was washed and then scanned for green fluorescence. The result showed exclusively binding of the C3G-labeled peptide on the spots where SH3 domain was chemoselectively immobilized (Figure 2a). The fluorescent C3G peptide bound to the SH3 microarray was quantified and showed a clear correlation between the amount of SH3 spotted and the fluorescence detected (Figure 2b).

In summary, we have demonstrated a new strategy for the site-specific immobilization of recombinant proteins onto a glass surface that can be used for the fabrication of protein microarrays. Our method is based on the chemoselective reaction between protein α-thioesters and a Cys-containing glass surface. The reaction results in a selective attachment of the protein to the glass surface through a stable amide bond. Recombinant protein α-thioesters are readily prepared using engineered protein splicing units.[3c,5,8,11] The approach reported in this work shows great promise for protein microarray preparation and high-throughput screening of biological interactions.

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Supporting Information Available: Results of fusion protein construction, linker synthesis, and experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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