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Intein-Mediated Biotinylation of Proteins and Its Application in a Protein Microarray

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DNA microarray is currently the method of choice for high-throughput analysis of nucleic acids at their transcriptional level. However, it has been shown that the mRNA expression level in a cell does not correlate well with the abundance of proteins.[1] To gain more insights into protein functions, a number of technologies[2] and binding chemistry[3] have been developed to immobilize small molecules,[3a,b] peptides,[3c,d] and proteins[3e−g] in a microarray for high-throughput protein studies.[4] In most cases, however, protein immobilization was achieved via their nucleophilic residues, resulting in random orientations of proteins on the glass surface. Thus far, there has only been one report of site-specific attachment of proteins on glass slides.[5] Approximately 6000 yeast proteins were expressed as his-tag fusions, spotted onto Ni-NTA functionalized slides, and >80% were found to retain their full biological activities, presumably as a result of site-specific immobilization which ensures most proteins on the slide to be oriented correctly. However, the binding between Ni-NTA and his-tag proteins is neither very strong nor very stable and susceptible to interference by many commonly used chemicals,[6] making this immobilization method incompatible with many protein screening assays. On the contrary, the biotin−avidin interaction, one of the strongest noncovalent interactions known,[7] is stable toward a variety of harsh conditions[8] and has been widely used in standard biochemical assays for immobilization purposes. The in vivo and in vitro biotinylations of proteins have previously been reported,[9] but with limited success due to low yields and the nonspecific nature of the biotinylation reaction.[9c] In some cases, biotinylation results in the addition of a long peptide to the target protein, which may interfere with proper folding of the protein.[9d] Furthermore, avidin is known to be toxic to cells, making the expression of avidin-fused proteins a difficult task.[10] Intein-mediated expression of proteins has mostly been developed for purification of fusion proteins on chitin columns[11] and has also found wide application in protein engineering where the expressed protein ligation (EPL) strategy is utilized.[11b,c] Recently, this strategy has been extended to modifications of proteins at their C-termini with a number of chemical tags.[12] We report here the first example of using an intein-mediated expression system to express, purify, and site-specifically biotinylate proteins, followed by immobilization onto avidin-functionalized glass slides. Thus, we have created a novel protein array on which site-specifically oriented proteins are immobilized with a highly robust and extremely stable linkage (Scheme 1) and are able to retain their full biological activities.

Three proteins of interest, namely MBP (maltose binding protein), EGFP (enhanced green fluorescent protein), and GST (glutathione S-transferase) were chosen as models and expressed in vivo as fusion proteins with an intein tag (intein fused to chitin binding domain) at their C-termini. The proteins were purified and biotinylated, in a single step, by first loading the crude cell lysate onto a column packed with chitin beads and then flushing the column with biotinylated cysteine, **1**, to obtain the C-terminally biotinylated proteins (Scheme 1), which were unambiguously confirmed by SDS-PAGE and western blotting (Figure 1). On the basis of SDS-PAGE, the biotinylation reaction took place with 90−95% efficiency, generating proteins in sufficient purity (>95%) which were spotted directly, without any further treatment, onto an avidin-functionalized slide to obtain the corresponding protein array.

A protein array was generated with the biotinylated EGFP, MBP, and GST and probed with Cy3-anti-EGFP, Cy5-anti-MBP, and

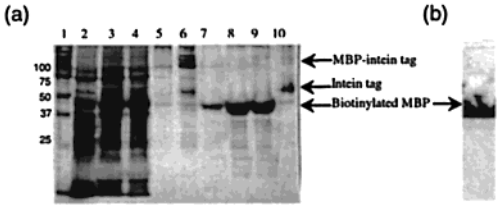
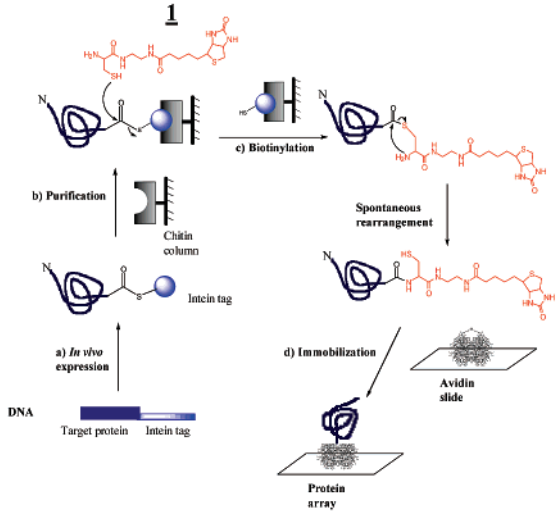


Figure 1. MBP purification and biotinylation. (a) (1) Protein marker, (2) uninduced cell extract, (3) induced cell extract, (4) flow-through from column loading, (5) flow-through from column wash, (6) proteins bound to chitin column before cleavage, (7) flow-through from quick flush of cleavage agent, (8−9) first two elution fractions after overnight incubation at 4 °C with **1**, (10) remaining proteins bound to chitin column after cleavage. (b) Western blotting of biotinylated MBP using Streptavidin-HRP for detection.

Scheme 1. Intein-Mediated Expression, Purification, Biotinylation, and Immobilization of Proteins



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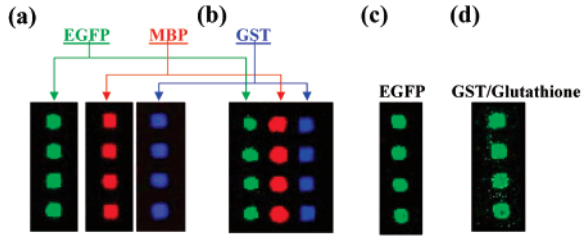

Figure 2. Site-specific immobilization of biotinylated, functionally active proteins onto avidin slides. (a) EGFP, MBP, and GST were individually detected with Cy3-anti-EGFP (green), Cy5-anti-MBP (red), and FITC-anti-GST (blue), respectively; (b) specific detection of all three proteins with a mixture containing all three antibodies; (c) fluorescence from the native EGFP; (d) specific binding between GST and its Cy3-labeled natural ligand, glutathione. No binding between glutathione and EGFP/MBP was observed (data not shown).

FITC-anti-GST, respectively. Three corresponding nonbiotinylated proteins were also spotted onto the same slide, as controls, and the array was incubated with either individual antibodies (Figure 2a) or a mixture of all three antibodies (Figure 2b). Only specific binding between the biotinylated proteins and their corresponding antibodies was observed, regardless of the presence of other proteins (Figure 2a) and antibodies (Figure 2b), indicating the specific immobilization and versatility of this new protein array. Furthermore, no fluorescence signal was observed with the nonbiotinylated control proteins (data not shown), confirming the essence of biotinylation for protein immobilization.

The most critical issue in generating a protein array is to ensure that proteins maintain their native activity, as it is previously known that proteins tend to denature on glass surfaces.[3e] To confirm that biotinylated proteins immobilized on the avidin slide retain their proper foldings, the native fluorescence of EGFP on the slide was monitored (Figure 2c). No loss of fluorescence intensity was observed after prolonged incubation at 4 °C, suggesting that folding of the protein was properly maintained on the slide. In a separate experiment, a slide immobilized with EGFP, MBP, and GST was incubated with Cy3-labeled glutathione, a known natural ligand of GST (Figure 2d). The result showed exclusive binding between GST and glutathione, further indicating full retention of the native GST activity. Furthermore, all data gathered thus far indicates that the presence of avidin as a molecular layer between the immobilized proteins and the glass surface also serves to minimize nonspecific absorption of proteins.[13a] Further improvement may be readily made by using streptavidin as the immobilization agent on the slide in place of avidin, which is a glycoprotein and known to have higher nonspecific binding characteristics.[14]

Thus far, the only reported method for site-specific attachment of proteins in a microarray has been the immobilization of his-tag proteins on slides functionalized with Ni-NTA.[5] However, the binding between his-tag proteins and the Ni-NTA complex is not very strong and is incompatible with many common chemicals such as DTT, SDS, EDTA, etc. The binding is also depleted outside the 4−10 pH range or when the buffer contains high concentrations of common salts. On the contrary, the binding between biotin and avidin is one of the strongest known (dissociation constant $\approx 10^{-15}$ M) and is stable under most stringent conditions.[7] Avidin is also extremely stable,[8] making it an ideal agent for slide functionalization. In addition, the interaction between avidin and biotin is instantaneous, hence requiring no incubation for protein immobilization. To confirm the benefit of the avidin−biotin linkage, slides immobilized with GST were first subjected to a number of harsh washing conditions and then detected with FITC-labeled anti-GST for any loss of GST on the surface. No loss of GST was observed even after the slide had been treated with (1) 1 M acetic acid at pH 3.3, (2) 60 °C water, and (3) 4 M GuHCl for a prolonged time (see Supporting Information), in sharp contrast with that of his-tag proteins on a Ni-NTA slide.[13b]

Findings described here present a new strategy for site-specific protein biotinylation and immobilization on a glass surface, generating a novel protein array on which proteins are oriented optimally and able to retain their native activity suitable for subsequent biological screenings. The advantage of avidin/biotin linkage over his-tag/Ni-NTA strategies for protein immobilization is highlighted by its ability to withstand a variety of chemical conditions, which may make this new protein array compatible with most biological assays.

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Supporting Information Available: Detailed protocols of protein expression, purification, biotinylation and immobilization, chemical synthesis of compound **1**, and microarray related experiments (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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