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Expanding the scope of site-specific protein biotinylation strategies using small molecules

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Abstract—We present a new approach to site-specifically biotinylate protein in a cell-free protein synthesis system with puromycin-containing small molecules. With this new method, biotinylated proteins were generated from the DNA templates in a matter of hours, making it useful for protein microarray generation. We also validated that the method is compatible with other high-throughput cloning/proteomics methods.

In the post-genome era, researchers are faced with a mammoth challenge—to develop new techniques capable of identifying and characterizing all proteins encoded by the human genome. One of the most promising technologies available is the protein microarray, which offers the platform for simultaneous studies of tens of thousands of proteins.[1] Despite numerous advances in recent years,[1a] the development of the protein microarray technology is still in its infancy, facing numerous and complex obstacles, one of which is to develop efficient methods for protein immobilization onto glass surfaces while maintaining their native biological functions. This is because proteins are 'delicate'—they may unfold and lose their activity if not properly attached to a suitable surface. A survey of the literature from the last few years reveals, however, that few immobilization strategies exist which allow for uniform and stable immobilization of proteins in a microarray.[2] Zhu et al. reported the first example of site-specific attachment of $(His)_6$-tagged proteins onto Ni–NTA-coated glass slides in their generation of the 'yeast proteome array'.[1b] However, the noncovalent $(His)_6$–Ni–NTA interaction is not very strong, often susceptible to interference by commonly used chemicals and salts, making this strategy useful to only a handful of downstream protein array applications. We and other have focused on developing alternative approaches which allow stable, and at the same time site-specific immobilization of proteins.[2] Mrksich and co-workers captured cultinase-fused proteins onto glass surfaces coated with a phosphonate ligand, achieving site-specific and covalent immobilization of the proteins.[2a] Johnsson and co-workers successfully developed a site-specific method to covalently immobilize hAGT-fused proteins onto modified glass surfaces.[2b] We developed intein-mediated approaches which allow site-specific biotinylation of proteins (both in vitro and in vivo) that may be subsequently spotted onto an avidin-functionalized glass slide to generate the corresponding functional protein microarray.[2c,d] To this end, we report a cell-free strategy which utilizes puromycin-containing small molecules to site-specifically biotinylate proteins at their C-termini.

Puromycin is an aminonucleoside antibiotic produced by *Streptomyces alboniger*. As puromycin resembles the 3′ end of the aminoacyl-tRNA, it competes with the ribosomal protein synthesis by blocking the action of the peptidyl transferase, leading to inhibition of protein synthesis on both prokaryotic and eukaryotic ribosomes. It was previously found that, at low concentrations, puromycin and its analogs act as noninhibitors of the ribosomal protein synthesis, and get incorporated at the C-terminus of the newly synthesized protein.[3] Our approach herein extends the strategy to protein microarray applications by exploiting a similar phenomenon (Fig. 1): by the use of a suitable amount of a

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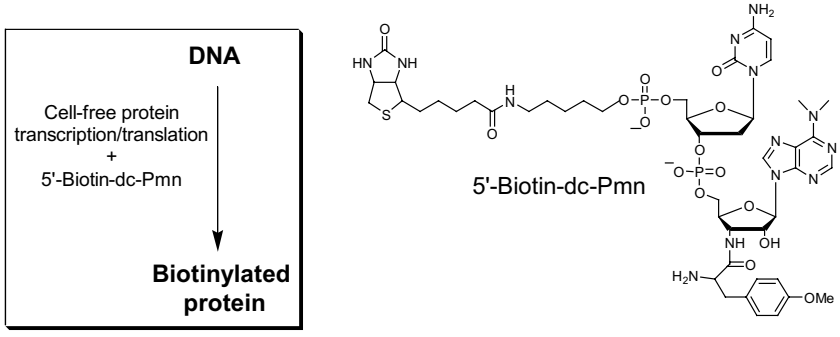

Figure 1. Cell-free protein expression/biotinylation.

puromycin-conjugated biotin molecule, 5′-Biotin-dc-Pmn, we demonstrated, for the first time, that proteins can be synthesized from a cell-free system, biotinylated at their C-termini and subsequently spotted onto an avidin-functionalized glass slide to generate the corresponding functional protein microarray. Using this newly developed method, biotinylated proteins could be obtained in a matter of hours using plasmids or PCR products as DNA templates. We further showed this method is compatible with other high-throughput cloning/proteomics methods such as the Gateway™ cloning strategy.

To demonstrate our strategy, 5′-Biotin-dc-Pmn was either conveniently synthesized using standard DNA synthesis protocols in house, or obtained from commercial sources (Dharmacon RNA Technologies, USA). Cell-free reactions were carried out with the plasmid DNA, GFP-pIVEX2.4Nde, as well as its PCR product, both carrying the GFP gene and regulatory elements needed for in vitro transcription/translation, to synthesize biotinylated GFP in the presence of differing amounts of 5′-Biotin-dc-Pmn (Fig. 2a and b, respectively).[4] Western blots with both anti-$(His)_6$ antibody (top gels) and anti-biotin antibody (bottom gels) were used to determine the overall GFP expression level, and the amount of biotinylated GFP produced in each reaction, respectively: with an increasing concentration of 5′-Biotin-dc-Pmn (0–60 μM), a concomitant decrease in GFP expression was evident, indicating the inhibitory property of puromycin (and its analogs) toward the ribosomal protein synthesis (top gels). On the other hand, the amount of biotinylated GFP first increased with increasing concentrations of 5′-Biotin-dc-Pmn (0–20/30 μM; lanes 1 to between 3 and 4 in bottom gels of Fig. 2a and b), then gradually decreased (lanes 4–5), suggesting that, while a high concentration of 5′-Biotin-dc-Pmn increased the yield of biotin incorporation into GFP, it also inhibited the overall expression of the protein. It should be noted that, our results agree well with what was observed previously.[3d] In some cases, low molecular-weight bands were also evident in the anti-biotin western blot, but not in the anti-$(His)_6$ western blot (labeled with * in Fig. 2a and b, bottom gel vs top gel, respectively), indicating the formation of truncated, biotinylated proteins as a result of pre-mature termination of protein synthesis due to the addition of the puromycin analog. An optimized concentration of 5′-Biotin-dc-Pmn (25 μM in a 25 μL cell-free reaction for

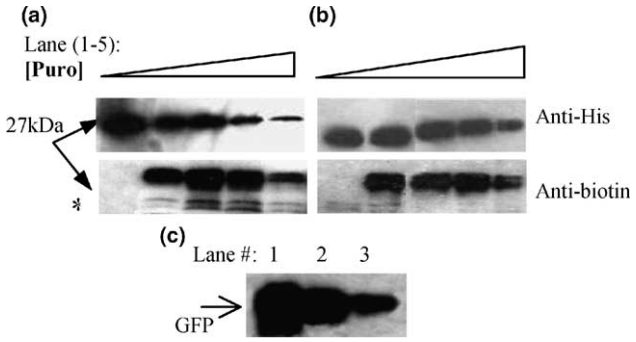

Figure 2. Puromycin-based protein biotinylation. Expression and biotinylation of GFP using (a) plasmid DNA, GFP-pIVEX2.4Nde and (b) PCR product as DNA template. Western blots with both anti-$(His)_6$ antibody (top gels) and anti-biotin antibody (bottom gels) were shown. Concentrations of 5′-Biotin-dC-Pmn in lanes # 1–5: 0, 10, 20, 30, and 60 μM. Suspected truncated, biotinylated proteins were labeled with *. (c) Efficiency of cell-free biotinylation of GFP proteins. The cell lysate was incubated with Neutravidin™ beads, and the resulting fractions were analyzed by western blots with anti-$(His)_6$ antibody to assess the degree of biotinylation. Lane 1: lysate before adsorption; lane 2: proteins absorbed onto the beads (biotinylated fraction); lane 3: lysate remained in solution (nonbiotinylated fraction). A separate western blot was done with anti-biotin antibody to ensure the successful separation of biotinylated/nonbiotinylated GFP in the absorption experiment (not shown).

RTS™ system) was determined to give the maximum amount of the full-length biotinylated GFP from the plasmid template (while minimizing side reactions).[4] Further optimizations of other parameters (e.g., DNA template concentration, incubation temperature, and reaction time) in the cell-free protein biotinylation reaction concluded that, best conditions in a 25 μL reaction were the following: 125 ng of plasmid DNA template, 25 μM of 5′-Biotin-dc-Pmn, 30 °C for 6–9 h using the RTS™ system. These conditions were thus used for all subsequent studies, unless indicated otherwise. Control reactions without addition of 5′-Biotin-dc-Pmn were performed (lanes 1 in Fig. 2a and b): no biotinylated GFP was detected in the reaction with either the plasmid or PCR DNA template, validating that our cell-free protein biotinylation strategy depends entirely on the addition of the puromycin-bearing small molecule.

We also investigated the protein biotinylation efficiency in our strategy, by comparing the amount of biotinylated protein synthesized (biotinylated GFP) versus the

amount of the total protein synthesized (biotinylated + nonbiotinylated GFP), as shown in Figure 2c. We estimated that at least 50% of the total proteins were synthesized in which >50% were successfully biotinylated. This gave a >25% overall biotinylation yield in our reaction, indicating that between 25 and 125 μg/mL of the biotinylated protein was produced. Again, these findings are in good agreement with previously reported results.[3d,e]

We next examined whether biotinylated proteins synthesized using our cell-free system could be used directly for protein microarray applications.[5] We used GFP-pIVEX2.4Nde plasmid as the DNA template, together with 5′-Biotin-dc-Pmn, in a cell-free reaction to generate the biotinylated GFP. A control lysate was obtained in which GFP was similarly expressed using the same cell-free system but without the addition of 5′-Biotin-dc-Pmn. Upon simple desalting steps following the reaction, the resulting crude lysate, containing newly expressed biotinylated GFP together with other nonbiotinylated proteins present in the cell lysate, was taken directly and spotted onto an avidin-functionalized glass side (lane 2 in Fig. 3). The control lysate was treated similarly and subsequently spotted on the same slide (lane 1 in Fig. 3): native fluorescence of GFP was observed with spots obtained from the biotinylated lysate, but not those obtained from the control lysate, demonstrating the feasibility, at the same time underscoring the importance, of using biotinylated proteins synthesized from cell-free systems for protein microarray generation.

The Gateway™ cloning strategy provides perhaps one of the most efficient means for high-throughput cloning and proteomics experiments.[6,7] One of the most essential components in Gateway™ cloning is the Destination vector, in which a target gene is cloned and subsequently expressed in a suitable host. In order to evaluate whether our cell-free protein biotinylation strategy is compatible with Gateway™ cloning, we constructed our own 'Destination vector', pDEST-IVEX2.4Nde, such that it is optimized for protein expression using the RTS™ system, thus compatible with our cell-free protein biotinylation strategy (Fig. 4a). Three proteins, namely MBP (maltose-binding protein), EGFP (enhanced green fluorescent protein), and GST (glutathione-*S*-transferase) were chosen as models and conveniently cloned into pDEST-IVEX2.4Nde Destination vector, following standard Gateway™ cloning protocols. The resulting constructs were used as DNA templates in our cell-free system to generate the corresponding biotinylated proteins in the presence of 5′-Biotin-dc-Pmn (25 μM). As shown in Figure 4b, all three proteins were successfully expressed and biotinylated. Few side products were generated in the reaction, which was consistent with our earlier cell-free biotinylation results.

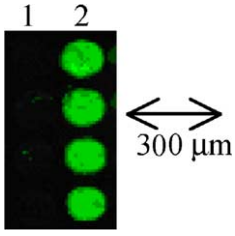

Figure 3. Generation of a functional protein array using biotinylated proteins. Lane 1: control lysate in which GFP was expressed without the presence of 5′-Biotin-dc-Pmn; lane 2: lysate in which GFP was expressed in the presence of 25 μm of 5′-Biotin-dc-Pmn.

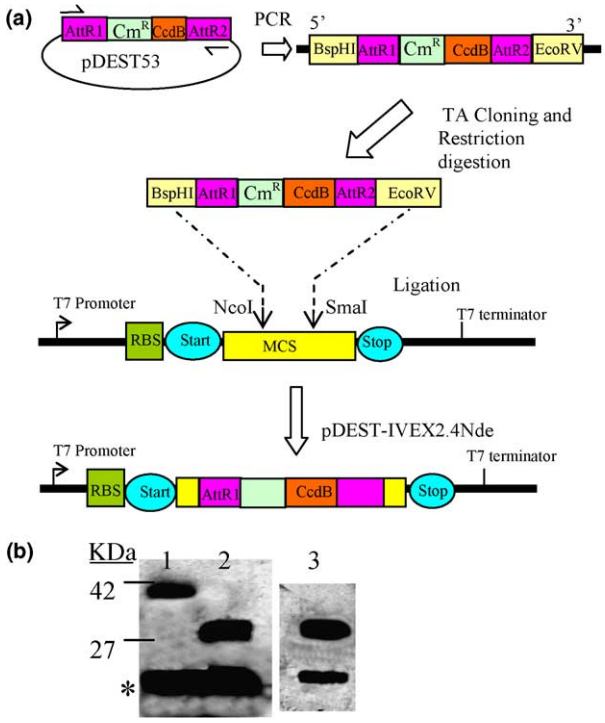

Figure 4. Compatibility of the Gateway™ Cloning System with the cell-free biotinylation strategy. (a) Schematic representation of the cloning of destination vector, pDEST-IVEX2.4Nde, suitable for the cell-free biotinylation strategy. (b) Anti-biotin blots showing the successful biotinylation of three model proteins cloned into pDEST-IVEX2.4Nde using Gateway™ cloning. Lane 1: MBP; lane 2: EGFP; lane 3: GST. Bands labeled with * were from 5′-Biotin-dC-Pmn.

In summary, we have developed a new strategy to efficiently biotinylate proteins in a cell-free protein expression system using puromycin-containing biotin analogs. The strategy is highlighted by its simplicity, in that protein biotinylation can be performed in a standard cell-free protein synthesis reaction, with the simple addition of a puromycin-containing biotin reagent, 5′-Biotin-dC-Pmn. No other modifications of the standard cell-free system are required. Cell-free protein synthesis provides an attractive alternative for potential high-throughput production of proteins. It enables minute amounts of proteins to be synthesized directly from their DNA templates (both plasmids and PCR products) in a matter of hours in 96- or 384-well formats. The strategy gave relatively good biotinylation efficiency (>50% of all synthesized proteins) and acceptable overall yield (>25% compared with the puromycin-free reaction). The potential application of our strategy was illustrated by the successful generation of a prototypical protein microarray using the biotinylated proteins obtained. We showed

the strategy is compatible with existing high-throughput cloning methods, such as the Gateway™ cloning technology. We are currently evaluating the generality of this strategy by applying it to the synthesis/biotinylation of a variety of other proteins.

Acknowledgements

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4. For discussions on the RTS™ system, see: www.roche.com. For protein expression and biotinylation, the plasmid containing the green fluorescent protein (GFP) with a $(His)_6$ tag and under the transcriptional control of T7 promoter, GFP-pIVEX2.4Nde (Roche), was used as the DNA template in a RTS™ 100 *E. coli* HY kit. Each reaction consists of 50–500 ng of DNA template, 6 μL of *E. coli* lysate, 5 μL of reaction tube, 6 μL of amino acids, 0.5 μL of 1 mM methionine, 2.5 μL of the reconstitution buffer. 5′-Biotin-dc-Pmn was added in different concentrations, ranging from 0 to 60 μM. The protein synthesis reaction was carried out at 30 °C for 6–9 h in a DNA Engine™ thermal cycler (MJ Research, USA). At the end of synthesis, the lysate was analyzed for protein expression/biotinylation with (1) fluorescence microplate reader (excitation wavelength: 395 nm; emission wavelength: 504 nm) to quantitate fluorescence readouts from the expressed GFP (data not shown), and (2) SDS-PAGE analysis and western blots. Western blots were done with horseradish peroxidase (HRP)-conjugated anti-biotin antibody, HRP-conjugated anti-His antibody (NEB), and the Enhanced ChemiLuminescent (ECL) Plus Kit (Amersham). The results were used to confirm the degree of GFP expression and biotinylation, respectively. The linear template DNA for the RTS reaction was generated with the RTS *E. coli*. Linear Template Generation Set™ following the vendor's instructions. Briefly, the PCR mixture (25 μL) contains 2.5 μL of 10× HotStar™ Taq DNA polymerase buffer (Qiagen), 0.2 mM of dNTPs (NEB), 1 μM each of the T7 promoter and terminator primer (Roche), 100 ng of GFP-pIVEX2.4Nde, and 2 units of HotStar™ Taq DNA polymerase (Qiagen). Amplification was carried out at 95 °C × 1 min, 60 °C × 1 min, and 72 °C × 1 min, for 30 cycles. The resulting PCR-generated, linear template (0.5 μL in each reaction) was used directly, without further purifications, in subsequent cell-free transcription/translation/protein biotinylation reactions using conditions similar to ones described earlier for the plasmid DNA. Similarly, western blot analysis with anti-biotin antibody, anti-His antibody, and the ECL Plus Kit were performed to confirm the presence of GFP expression and biotinylation.
5. At the end of the cell-free protein expression/biotinylation using the puromycin method, the lysate (25 μL) was passed through a G25 microspin column (Amersham) to remove most of the residual 5′-Biotin-dc-Pmn. The eluted product (in PBS) was taken, spotted directly onto an avidin-functionalized glass slide and subsequently processed as previously described.[1] The spotted slide was washed thoroughly with PBST (0.1% Tween in PBS) to remove any nonbiotinylated proteins, then visualized for native GFP fluorescence using an ArrayWoRx™ microarray scanner (Applied Precision, USA). In order to confirm that the single-step immobilization/purification method removes nonbiotinylated impurities, the crude lysate was first spiked with a pure protein (GST, nonbiotinylated), spotted onto the avidin slide, washed thoroughly, and detected with anti-GST. As expected, no GST binding was observed on the slide (data not shown).
6. For discussion on the Gateway™ cloning, see: www.invitrogen.com.
7. For recent key examples of Gateway™ cloning, see: (a) Ho, Y. et al. *Nature* **2002**, *415*, 180–183; (b) Gavin, A. et al. *Nature* **2002**, *415*, 141–147.