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Versatile Protein Biotinylation Strategies for Potential High-Throughput Proteomics

Rina Y. P. Lue,[†] Grace Y. J. Chen,[†,‡] Yi Hu,[†] Qing Zhu,[†] and Shao Q. Yao*[,†,‡]

Contribution from the Department of Biological Sciences, and Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Republic of Singapore

Received August 13, 2003; E-mail: chmyaosq@nus.edu.sg

Abstract: We present intein-mediated approaches for efficient biotinylation of proteins site-specifically. The reactive C-terminal thioester generated from intein-assisted protein splicing (either in vitro or in live cells) served as an attractive and exclusive site for attaching cysteine-containing biotin. Using these novel biotinylation strategies, we were able to efficiently biotinylate many proteins from different biological sources in a potentially high-throughput, high-content fashion. Some of these proteins were subsequently immobilized, in a very simple manner, onto different avidin-functionalized solid surfaces for applications such as protein microarray and surface plasmon resonance (SPR) spectroscopy, highlighting the numerous advantages of using biotin over other tags (e.g., GST, His-tag, etc.) as the method of choice in protein purification/immobilization. In addition, our intein-mediated strategies provided critical advantages over other protein biotinylation strategies in a number of ways. For the first time, we also successfully demonstrated that intein-mediated protein biotinylation proceeded adequately inside both bacterial and mammalian living cells, as well as in a cell-free protein synthesis system. Taken together, our results indicate the versatility of these intein-mediated strategies for potential high-throughput proteomics applications. They may also serve as useful tools for various biochemical and biophysical studies of proteins both in vitro and in vivo.

Introduction

Proteomics is an emerging field aiming to identify and characterize all protein complements in the cell.[1] One of the most promising technologies in proteomics is the protein microarray, which offers the possibility to simultaneously study most proteins expressed in an organism. Already, this has been realized in *Saccharomyces cerevisiae*, where more than 90% of proteins expressed by the yeast ORFs were immobilized on a single 25 × 75 mm glass slide to generate a yeast proteome array.[2] To accomplish this mammoth task, the authors utilized a double-tagging system to laboriously express proteins in the form of fusions containing both $(His)_{6-}$ and GST tags, purify then using the glutathione (GSH) column, and subsequently immobilize them onto a Ni−NTA coated glass slide to generate the proteome array. This strategy, however, has a number of drawbacks: (1) the entire process is extremely tedious, requiring multiple steps of manipulation; (2) protein immobilization using His-tag/Ni−NTA interaction is neither strong nor robust enough, limiting the protein array to ONLY downstream applications where mild conditions are used; (3) the use of a macromolecular tag such as GST (M_w > 25 KDa), which has moderate affinity to glutathione resin, may not only affect the structure and activity of the native protein, but also (**4**) limit the strategy to in vitro-based applications in simpler organisms (e.g., yeast), where background bindings from nonspecific protein sources are much lower and thus require only simple, nonstringent washings. Consequently, there is an urgent need to develop alternative methodologies that address these issues.

Since the 1980s, avidin−biotin technology has gained much prominence in research due to the remarkable affinity between avidin (or streptavidin, its bacterial relative from *Streptomyces avidinii*) and biotin (vitamin H, 0.24 kDa).[3] With a K_d of 10^{-15} M, avidin/biotin binding is the strongest noncovalent interaction known in nature. Consequently, avidin/biotin systems have been exploited for a variety of diverse applications in modern biology.[3−4] Historically, biotinylation of proteins has been carried out by standard bioconjugate techniques using biotin-containing chemicals. This leads to random biotinylation of proteins and in many cases, the subsequent inactivation of their biological activities.[5] Alternative techniques have been developed which allows for site-specific labeling of proteins with biotin.[6−9] Minimal amino acids sequences were identified by Cronan for site-specific tagging of proteins,[7] in which the covalent attachment of biotin to specific lysine residue was catalyzed by biotin ligase (EC 6.3.4.10), a 35.5 kDa monomeric

[†] Department of Biological Sciences.
[‡] Department of Chemistry, National University of Singapore.

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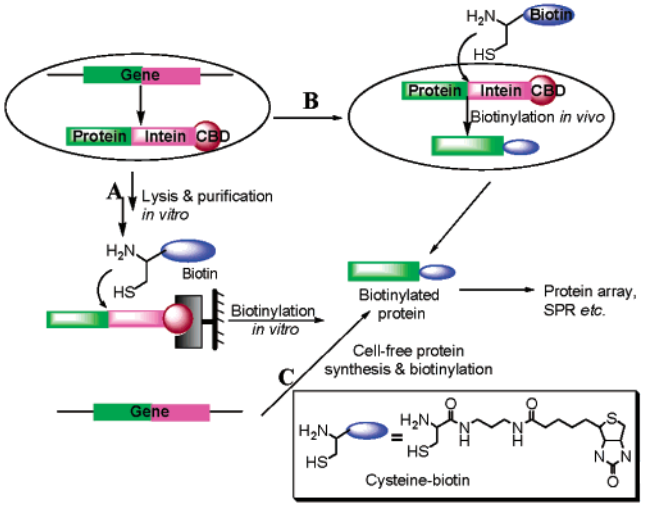
enzyme encoded by the *birA* gene.[8] These sequences, however, are typically quite large (>63 AAs) and thus may interfere with the biological activity of the proteins to which they are fused. Further optimizations of these sequence tags revealed that smaller tags (15−30 AAs) may be used.[9] In general, proteins fused with these tag sequences are biotinylated either in vitro or in vivo by biotin ligase. Unfortunately, in vivo biotinylation of proteins catalyzed by biotin ligase is often inefficient and toxic to cells due to a decrease in biotinylation of important endogenous proteins within the host cells.[10,11] Recent advances in the field, however, have partially rectified this problem, and at the same time unequivocally demonstrated numerous advantages associated with protein biotinylation in live cells.[12] In vitro biotinylation is used when in vivo expression of the soluble fusion protein is insufficient. This, however, also faces with problems such as proteolytic degradation of tag sequences and inhibitory effects of commonly used reagents toward biotin ligase.[10]

Intein was first discovered in *Saccharomyces cerevisiae* as a protein splicing element.[13] To date, over 100 different inteins have been identified from different organisms.[14−15] Elucidations of the protein splicing mechanism in inteins have led to the design of "engineered" inteins that perform single splice-junction cleavage under specific conditions. These inteins, upon fusion to a protein (either at its C or N terminus), may lead to the generation of a reactive C-terminal thioester or an N-terminal cysteine, respectively.[16,17] The unique chemical properties of N-terminal cysteine/C-terminal thioester in the protein have been exploited in protein engineering to create proteins having unnatural functionalities using the so-called native chemical ligation.[18−21] We recently described a novel approach for site-specific biotinylation of proteins in vitro using the intein fusion system.[22] We showed the strategy could be used to incorporate biotin exclusively at the C-terminus of proteins, which may then be immobilized onto an avidin-coated glass slide to generate a protein microarray. To this end, we report new findings derived from this strategy and their potential applications in high-throughput proteomics. We reason that, for the purpose of proteomics applications, especially in the case of protein microarray, the high affinity of biotin/avidin interaction may allow facile purification and subsequent downstream applications of the biotinylated proteins under highly stringent conditions. There are few naturally biotinylated proteins, meaning background protein bindings which are otherwise problematic with most types of affinity processes may be minimized.[12] All proteins are uniformly oriented on the glass surface via biotin/avidin interaction, thus ensuring they are functionally accessible.[22,23] Furthermore, biotinylation in our approach does not require introduction of any extra tag to the final protein, thus minimizing any potential perturbation to the protein function, making this method attractive for future in vivo-based applications.

Scheme 1. Three Intein-Mediated Protein Biotinylation Strategies: (A) In Vitro Biotinylation of Column-Bound Proteins; (B) In Vivo Biotinylation in Live Cells; (C) Cell-Free Biotinylation of Proteins.



Our strategy is summarized in Scheme 1. In our previous work with 3 model proteins,[22] we demonstrated that site-specific biotinylation of proteins could be efficiently carried out by applying a cysteine-containing biotin tag to the intein-fused protein purified and bound on a chitin column (method A in Scheme 1). Since both cleavage/elution and biotinylation of the protein were carried out in a single step, and the eluted fraction could be directly immobilized on an avidin-coated surface without any further purification, the method is well suited for efficient generation of a large number of ready proteins for protein array and other applications. We now validate this and present new findings. Specifically, we show the following: (1) the strategy may be used to express a variety of proteins from different organisms with ease and high efficiency (method A in Scheme 1); (2) for the first time, the intein-mediated biotinylation strategy is successfully implemented in live cells (both bacterial and mammalian) to generate biotinylated proteins; (3) which could be subsequently taken to generate the corresponding protein array in a single step without any purification (method B in Scheme 1); and (4) the strategy may be extended to protein biotinylation in a cell-free synthesis system (method C in Scheme 1).

Materials and Methods

General. Chitin resin, pTYB1 and pTYB2 expression vectors were purchased from New England Biolabs (NEB). pTYB1 and pTYB2 enable expression and isolation of proteins processing a C-terminal thioester. The target gene is inserted into the polylinker region of each vector, giving rise to the target protein fused in frame to the N terminus of the *Sce* VMA intein. The only difference between the two vectors lies within the 3′ end restriction site, just before the start of the intein gene. pTYB1 and pTYB2 contains Sap I and Sma I sites at their 3′ ends, respectively. The use of Sap I site in pTYB1 allows the C-terminus

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of the target protein to be fused directly next to the intein cleavage site, whereas the use of Sma I site in pTYB2 adds an extra glycine residue to the C-terminus of the target proteins. pT-Rex-DEST30 mammalian expression vector and yeast ex-clones were from Invitrogen (USA). Cysteine-biotin was prepared as previously described.[22] BIAcore X instrument and CM5 sensor chip used in SPR experiment were from Biacore (Sweden). MESNA, cell beads for cell lysis, avidin, and Dulbecco's modified Eagle's medium (DMEM) basal medium for cell culture were from Sigma. Avidin-functionalized glass slides were prepared as described previously.[22,23] Anti-MBP and anti-GST antibodies were from Santa Cruz Biotechnology (USA). Cy5 dye ($\lambda_{Ex} = 633$ nm; $\lambda_{Em} = 685$ nm) was from Amersham Biosciences (USA). FITC dye (($\lambda_{Ex} = 490$ nm; $\lambda_{Em} = 528$ nm) was from Molecular Probes (USA). Fetal calf serum and antibiotics were from Biological Industries (USA), and tissue culture plates were from Greiner (Germany). Other standard chemicals/biochemicals were purchased from their respective commercial sources, as indicated below.

Biotinylation of EGFP Mutants Having Different C-Terminal Residues. All pTYB-1 derived plasmids, including the plasmid coding for the wild-type EGFP fused to an intein, pTYB1-*wt*EGFP (Lys^{239})-intein, were constructed based on NEB's protocols and as previously described.[22] The C-terminal residue of *wt*EGFP in pTYB1-*wt*EGFP (Lys^{239})-intein was site-mutagenized from the original Lys^{239} to the other 19 amino acids using QuickChange XL Site-Directed Mutagenesis Kit (Stratagene). Briefly, 19 sets of primers, each containing a primer (5′-GAC GAG CTG TAC NNN TGC TTT GCC AA-3′) and a complementary primer (5′-TT GGC AAA GCA N′N′N′ GTA CAG CTC GTC-3′), were used, in which NNN (and N′N′N′) in each set of primers represents an amino acid to which Lys^{239} in pTYB1-*wt*EGFP (Lys^{239})-intein was replaced. Upon confirmation by DNA sequencing, the mutated plasmids (e.g., pTYB1-*mut*EGFP (AA^{239})-intein, where AA represents a corresponding mutated amino acid) were transformed into ER2566 *E. coli*. Protein expression and purification were performed as previously described.[22] Briefly, upon harvest and lysis, the clear supernatant was incubated with chitin resin for 30 min at 4 °C with gentle agitation. Subsequently, the resin was washed with the column buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA) followed by incubation with the cleavage buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA, 30 mM MESNA and 1 mM cysteine-biotin) overnight at 4 °C. Addition of MESNA was shown previously to promote intein-mediated ligation.[24] Upon resin settlement, the supernatant which contains the eluted, biotinylated protein was collected and may be used directly without further purifications. If desired, the eluted fraction could also be passed through a NAP-5 desalting column (Amersham) before use. Resin-bound proteins were analyzed by first boiling the resin with DTT-free SDS-PAGE loading buffer, then separated by SDS-PAGE and stained with Coomassie blue. Premature in vivo cleavage and on-column cleavage/biotinylation of the intein-fusion was determined from the stained SDS-PAGE gel (see the Supporting Information). To determine the ratio between the biotinylated and the nonbiotinylated protein in the eluted fraction, an absorption experiment with streptavidin beads was performed (see ref 22 and the Supporting Information). Briefly, the eluted fraction was incubated with excessive streptavidin magnetic beads for 1 h at 4 °C to ensure all biotinylated proteins were absorbed onto the beads. Both eluents, before and after streptavidin adsorption, were then analyzed by SDS-PAGE. Western blots with horseradish peroxidase (HRP)-conjugated anti-biotin antibody (NEB) and the Enhanced Chemi-Luminescent (ECL) Plus kit (Amersham) were performed to confirm the presence of biotin-tagged proteins. EGFP (Asp^{239})-intein and EGFP (Cys^{239})-intein were cloned into pTYB-2 vector via Nde I and Sma I site based on Impact-CN protocols (NEB).

High-Throughput Yeast Protein Expression and Biotinylation. All high-throughput yeast work was performed in 96-well formats wherever possible. To construct intein-fused yeast proteins, 96 different yeast genes were first PCR amplified from the yeast ex-clones (Invitrogen), and cloned into pTYB1. A common upstream primer (5′-GC GGC GGC CAT ATG GAA TTC CAG CTG ACC ACC-3′) containing an Nde I site with a translation initiation codon (ATG), and individual downstream primers (5′-GGC GGC TGC TCT TCC GCA ACC ACC N_{15-18}-3′) containing a Sap I site, were used in the PCR reaction to remove the stop codon and at the same time introduce 2 extra Gly residues to the C-terminus of the yeast gene. A standard PCR mixture (25 μL) contained 2.5 μL of 10× HotStarTaq DNA polymerase buffer (Qiagen), 0.2 mM of each dNTPs (NEB), 0.5 μM of each primer, 100 ng of plasmid DNA template and 2 units of HotStarTaq DNA polymerase (Qiagen). Amplification was carried out with a DNA Engine thermal cycler (MJ Research) at 94 °C for 45 s, 55 °C for 45 s and 72 °C for 2 min, for 25 cycles. The PCR products were cloned into pCR2.1-TOPO using TOPO TA cloning kit (Invitrogen) prior to double digestion with Nde I and Sap I (NEB). Digested yeast gene fragments of correct sizes were gel-purified and cloned into the pTYB1 vector via Nde I and Sap I sites to yield intein-fused constructs with two additional Gly residues at the cleavage site for improved biotinylation efficiency (see the Results and Discussion). Upon confirmation by DNA sequencing, the resulting plasmids were transformed into ER2566 *E. coli*. (NEB), grown in Luria Bertani (LB) medium supplemented with 100 μg/mL of ampicillin at 37 °C in a 250 rpm shaker to an OD_{600} of ~0.6, then induced overnight at room temperature using 0.3 mM isopropyl thiogalactosidase (IPTG). Upon harvest (4000 rpm, 15 min, 4 °C), cells were resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 1 mM EDTA, 1% CHAPS, 1 mM TCEP, and 1 mM PMSF) and lysed by glass beads (Sigma). The clear lysate was collected by centrifugation, loaded onto a microspin columns pre-packed with 100-μL chitin resin & preequilibrated with 1 mL of column buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl and 1 mM EDTA). To purify the fusion protein, the clear cell lysate was incubated on the column for 30 min at 4 °C with gentle agitation to ensure maximum protein binding. Unbound impurities were then washed away with 2 mL of column buffer. For biotinylation of yeast proteins, 200 μL of the column buffer containing 100 mM MESNA and 5 mM cysteine-biotin was passed through the column to distribute it evenly throughout the resin before the flow was stopped and the column was incubated at 4 °C overnight. The resulting biotinylated protein was eluted with 100 μL of column buffer, and analyzed by 15% SDS-PAGE gel. The whole protein expression process was monitored by SDS-PAGE, and the biotinylation of yeast proteins was unambiguously confirmed by western blots.

SPR Analysis. All SPR experiments were performed with a BIAcore X instrument. Biotinylated MBP was prepared as described above. Surface activation of the CM5 sensor chip was done using standard amino-coupling procedures according to manufacture's instructions. 1.75 μg of avidin in 10 nM acetate (pH 4.5) and 0.125 M NaCl was passed over the activated chip surface. Excessive reactive groups were then deactivated with 1 M ethanolamine hydrochloride (pH 8.5) before injection of 35 μL biotinylated MBP (10 μg/mL) to the avidin-functionalized surface. Subsequently, 10 μL of anti-MBP antibody (0.1 mg/mL) was injected at a flow rate of 1 μL/min to confirm the immobilization of MBP onto the chip surface. 10 mM HCl was used to regenerate the chip surface before subsequent rounds of antibody injections. The K_d of the anti-MBP/MBP binding was determined by BioEvaluation software installed on the BIAcore X.

In Vivo Protein Biotinylation in *E. coli*. For in vivo biotinylation of proteins in *E. coli*, pTYB-1 constructs containing MBP and two yeast proteins (YAL012W & YGR152C) were used. Liquid cultures of ER2566 carrying the genes were growth to OD_{600} of ~0.6 in LB medium supplemented with 100 μg/mL of ampicillin. Expression of MBP and yeast protein fusions was induced with 0.3 mM IPTG at 30 °C for 3 h and at room temperature overnight, respectively. MESNA and cysteine-biotin were subsequently added to final concentrations of

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30 mM and 3 mM, respectively. Other concentrations of MESNA/cysteine-biotin were also tested but these conditions gave the best in vivo bintinylation efficiency while maintaining the viability of cells. In vivo biotinylation was allowed to proceed overnight at 4 °C with gentle agitation. Cells were harvested and washed thoroughly with PBS to remove excessive MESNA/cysteine-biotin before lysed with glass beads. Clear lysates containing the desired biotinylated proteins were collected by centrifugation, and used without further purifications. The entire process was monitored by SDS-PAGE and western blots. In vivo protein biotinylation was unambiguously confirmed with HRP-conjugated anti-biotin antibody. Additionally, to confirm the affinity of the in vivo biotinylated protein toward avidin/streptavidin, and to determine the ratio of the biotinylated/nonbiotinylated proteins generated in vivo, an absorption experiment with streptavidin beads was performed (for details, see the previous paragraphs and the Supporting Information). Briefly, clear cell lysates were incubated with excessive Streptavidin MagneSphere Paramagnetic Particles (Promega) at 4 °C for 30 min. The beads were then thoroughly washed with PBS to remove unbound proteins, and subsequently analyzed by boiling in SDS-PAGE loading buffer, then resolved on a 12% SDS-PAGE gel, followed by immunoblotting with HRP-conjugated anti-biotin antibody. Cell lysates before and after streptavidin absorption were also separated on a 12% SDS-PAGE gel followed by western blots with anti-MBP and anti-biotin antibodies.

In Vivo Protein Biotinylation in Mammalian Cells. EGFP-intein was cloned into pT-Rex-DEST30 (Invitrogen) mammalian expression vector using Gateway cloning technology. HEK 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/mL). Cells were seeded at 2.4×10^6 cells per 100 mm tissue culture plate. After overnight incubation at 37 °C, cells were transiently transfected with the vector encoding EGFP-intein using PolyFect Transfection Reagent (Qiagen). After 48 h of expression, the culture medium was changed to DMEM containing 10 mM MESNA and 1 mM cysteine-biotin, and further incubated at 37 °C overnight. These biotinylation conditions were optimized to ensure cell viability and maximum biotinylation efficiency. Mammalian cells were then harvested, washed thoroughly with PBS to remove excessive biotin, and lysed by glass beads. The entire biotinylation process was monitored by SDS-PAGE and western blots (with anti-biotin antibody). The biotinylated protein in the mammalian cell lysates was purified using Streptavidin MagneSphere Paramagnetic Particles before unambiguously confirmed by immunoblotting using HRP-conjugated anti-biotin antibody as described earlier.

Protein Microarray Generation. All protein microarray work was performed as previously described,[22] with the following modifications. EGFP, GST, and MBP were biotinylated in live bacterial cells as described above. 10 mL of bacterial cell cultures were harvested and washed thoroughly with PBS before lysed with 100 μL of lysis buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 1 mM EDTA). The clear cell lysate containing the desired biotinylated protein was spotted directly onto an avidin-functionalized glass slide and subsequently processed as previously described.[22] The spotted slides were washed thoroughly with PBST (0.1% Tween in PBS) to remove any nonbiotinylated proteins, then incubated with a suitable fluorescently labeled antibody for 1 h before washing and scanning with an ArrayWoRx microarray scanner (Applied Precision). 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).

Cell-Free Synthesis and Biotinylation of MBP. The pTYB-1-MBP-intein plasmid was used as the DNA template in the Rapid Translation System (RTS) 100 *E. coli* HY kit (Roche) for cell-free protein synthesis. On the basis of the manufacturer's protocol, the reaction was performed at 30 °C for 4 h in a 25 μL reaction with 500 ng DNA as the template. At the end of protein synthesis, MESNA and cysteine-biotin were added to the lysate to final concentrations of 100 mM and 5 mM, respectively, to induce cleavage/biotinylation of MBP at 4 °C overnight. Cell lysates were precipitated with acetone and analyzed by SDS-PAGE. Biotinylation of MBP was unambiguously confirmed by western blots with HRP-conjugated anti-biotin antibody.

Table 1. Influence of C-Terminal Residues on the in Vivo Cleavage of EGFP-Intein and On-Column EGFP Cleavage/Biotinylation[a]

C-terminal residue of EGFP	In vivo cleavage	on-column cleavage/biotinylation
Ala	+	+++
Arg	+++	+++
Asn	+	+
Asp	100%	N. D.
Cys	+	N. D.
Gln	+	+++
Glu	++++	N. D.
Gly	+	++++
His	+++	+++
Ile	+	+
Leu	++	++
Lys	++	++++
Met	++	+++
Phe	++	++++
Pro	+	++
Ser	+	++
Thr	+	+++
Trp	++	+++
Tyr	+++	+++
Val	+	+

[a] N.D. = Not detected (e.g. 0% cleavage/biotinylation), (+) = < 25% cleavage/biotinylation, (++) = 25−50% cleavage/biotinylation, (+++) = 50−75% cleavage/biotinylation and (++++) = 75−100% cleavage/biotinylation.

Results and Discussion

Influence of C-Terminal Residues on in Vitro Biotinylation. The final yield of an in vitro biotinylated protein is primarily dependent upon the amount of the intein fusion recovered from cell extract and its subsequent on-column cleavage/biotinylation efficiency. It was previously reported that the C-terminal amino acid residue of the fused protein at the intein cleavage site greatly affects the cleavage efficiency of the intein.[24] We would like to have an in vitro system in which biotinylation is independent of the C terminus of proteins (method A, Scheme 1). We therefore examined the influence of C-terminal residue of the fused protein on its biotinylation. EGFP was cloned into pTYB1 expression vector to generate pTYB1-*wt*EGFP (Lys^{239})-intein, which contains EGFP fused to the intein tag via the original C-terminal residue of EGFP, Lys^{239}. Site-directed mutagenesis was subsequently performed to mutate Lys^{239} to the other 19 amino acids. The intein-fused proteins were overexpressed in *E. coli.*, and their in vivo cleavage before cell lysis was assessed. Results are summarized in Table 1 (column 2 and the Supporting Information). SDS-PAGE analysis showed that acidic amino acids (e.g., Asp and Glu) at the C-terminus of EGFP caused almost complete premature cleavage (~100%) of the EGFP-intein fusion protein inside the bacteria, whereas some other residues (e.g., Arg, His, and Tyr) caused substantial in vivo cleavage (>50%). The majority of C-terminal residues, however, caused less in vivo cleavage (<50%), thus allowing sufficient amounts of fusion proteins to be obtained prior to subsequent on-column cleavage/

biotinylation. Following cell lysis, the fusion protein was first bound to the chitin resin and their on-column cleavage/biotinylation efficiency was subsequently assessed by incubating the resin-bound protein with cysteine-biotin in the presence of MESNA. By streptavidin adsorption experiments (see the Materials and Methods and the Supporting Information) with selected proteins, it was determined that >95% of biotinylated proteins were consistently obtained in the eluted fractions following cysteine-biotin/MESNA treatments. Consequently, the amount of on-column protein cleavage (see the Supporting Information) was taken to quantitate the relative efficiency of protein biotinylation for respective EGFP mutants (column 3 in Table 1): most amino acids substituted at the cleavage site retained relatively high degrees of protein biotinylation (> 50%), whereas some other residues (e.g., Asn, Cys, Ile, and Val) generated relatively lesser amounts of biotinylated protein (<25%). No biotinylation was detected for EGFP mutants having Asp, Glu and Cys substituted at the cleavage site of the fusion (see Table 1 and the Supporting Information).

In order for our strategy to be generally applicable for biotinylation of proteins in high-throughput proteomics applications, the "C-terminal effect" on protein biotinylation needs to be minimized. On the basis of above mutagenesis experiments with the EGFP-intein fusion (Table 1), it was observed that having a Gly residue at the cleavage site minimized the premature cleavage of the fusion in the bacterial cells, and at the same time maximizing the subsequent on-column cleavage/biotinylation efficiency. We therefore reasoned that insertion of one or two extra Gly residues at the C terminus of a protein having undesired cleavage-site residues (e.g., Asp and Glu) should optimize protein biotinylation while introducing negligible effect on the protein function. We therefore cloned two EGFP mutants (i.e., EGFP(Asp^{239}) and EGFP(Cys^{239})), containing C-terminal Asp and Cys, respectively, into the pTYB2 vector. The resulting constructs, i.e., pTYB2-EGFP(Asp^{239})-intein and pTYB2-EGFP(Cys^{239})-intein, were the same as their pTYB-1 counterparts with the addition of an extra Gly at the C-terminus of each mutant. Protein expression from the new constructs revealed that (Figure 1A), when compared with the original PTYB1 constructs (the Supporting Information), addition of the extra Gly indeed substantially lowered the in vivo cleavage of the fusion protein [i.e., 70% for pTYB-2 construct vs 100% for pTYB-1 construct of EGFP(Asp^{239})-intein mutant]. Significantly improved biotinylation efficiency of the protein [i.e., up ~80% for pTYB-2 construct vs 0% pTYB-1 construct of EGFP(Cys^{239})-intein mutant; see Figure 1A] was also observed, thereby validating our hypothesis. Consequently, extra Gly residues were introduced in all of our subsequent experiments (vide infra).

High-Throughput Expression and Biotinylation of Yeast Proteins. To validate our in vitro biotinylation strategy for potential high-throughput protein expression, we cloned ~100 different yeast proteins in the form of intein fusions. Yeast proteins were chosen in our studies as their DNA sources are readily available. Two extra Gly residues were conveniently introduced at the C-terminus of each yeast protein by PCR to maximize biotinylation efficiency, and at the same time minimize pre-mature cleavage of the fusion protein in vivo. We found the cloning/protein expression/biotinylation could be readily adopted in 96-well formats, thus enabling high-

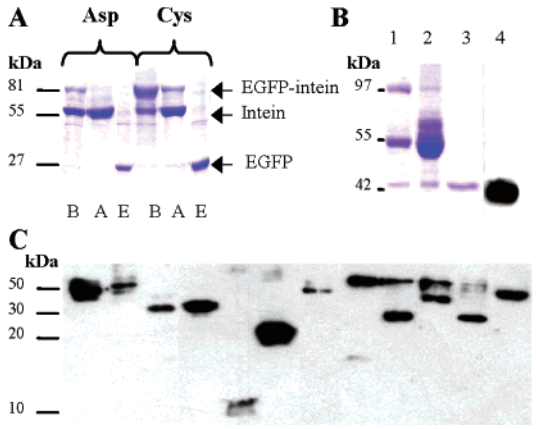

Figure 1. In vitro biotinylation of column-bound proteins. (A) Effect of an extra glycine residue on intein-mediated biotinylation. pTYB2-EGFP(Asp^{239})-intein and pTYB2-EGFP(Cys^{239})-intein were expressed, extracted and incubated with chitin beads. After washing, bound proteins were incubated with cysteine-biotin/MESNA. **B**: Proteins bound on chitin beads before cysteine-biotin elution; **A**: proteins remaining on chitin beads after cysteine-biotin elution; and **E**: eluted biotinylated EGFP. Coomassie blue staining of the SDS gel was presented. For data obtained with pTYB1-EGFP(Asp^{239})-intein and pTYB1-EGFP(Cys^{239})-intein, see the Supporting Information. (B) Purification and biotinylation of a yeast protein (YAL012W). Lane 1: proteins bound to chitin beads before cysteine-biotin elution; lane 2: remaining proteins bound to chitin beads after cysteine-biotin elution; lane 3: eluted biotinylated yeast protein; lane 4: immunoblot of lane 3 using anti-biotin antibody. (C) High-throughput expression and biotinylation of yeast proteins. Only 12 proteins (biotinylated fractions) were shown were on the immunoblot (detected with anti-biotin antibody).

throughput generation of potentially large numbers of proteins. Roughly half of the clones (~50) were further expressed, and 31 of which were successfully biotinylated (Figure 1B,C; only 12 were shown in Figure 1*C*). The remaining ones (~20) failed to express as soluble proteins in *E. coli.* and were not pursued further. As shown in Figure 1B, despite the introduction of 2 extra Gly residues at the C-termini of some yeast proteins, a substantial amount (~70%) of in vivo cleavage was still observed in the cell lysate, suggesting that alternative approaches may be explored in future to further rectify this problem. Fortunately, for most proteins, we were able to isolate sufficient amounts of the fusion proteins. In most cases, subsequent on-column cleavage/biotinylation steps typically eluted the desired biotinylated proteins as the predominant products with acceptable yields (Figure 1B,C). Variable degrees of protein biotinylation were observed for the yeast proteins (Figure 1C), which might have been caused by a number of different factors, including differences in the expression level of different yeast proteins, the extent of in vivo self-cleavage and different degrees of on-column cleavage/biotinylation, etc. Of the 31 biotinylated yeast proteins, many are yeast enzymes, covering a wide rage of biological activities (i.e., 4 kinases, 4 dehydrogenases, 4 phosphatases, 2 transferases, 2 lyases, 1 protease, 14 others) and molecular weights (i.e., 10−60 KDa), and further validating the generality of our biotinylation strategy.

Immobilization of Biotinylated Proteins onto Self-Assembled Monolayers (SAM) in SPR Experiments. Having demonstrated the feasibility of our intein-mediated strategy for high-throughput generation of biotinylated proteins, we next examined its biochemical applications. We previously showed that biotinylated proteins could be spotted directly onto an avidin-coated glass slide to generate a functional protein array.[22] Herein, we showed they may also be immobilized onto

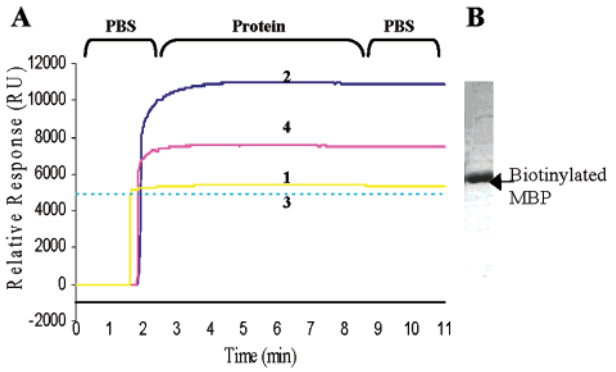

Figure 2. (A) SPR analysis of biotinylated MBP on an avidin-functionalized sensor chip. **1**: resonance response after injecting biotinylated MBP; **2**: resonance response after passing anti-MBP antibody (0.1 mg/mL) through the MBP-coated sensor chip; **3**: new baseline after 10 mM HCl regeneration; **4**: second round of antibody injection. (B) SDS-PAGE of purified MBP used in (A) stained with Coomassie blue.

other surfaces, such as that of self-assembled monolayers (SAM). We used Surface Plasmon Resonance (SPR) spectroscopy to follow the immobilization of biotinylated proteins onto an avidin-functionalized SAM surface. SPR allows direct visualization of protein immobilization in real time, as well as its subsequent interaction with other proteins.[25] Maltose binding protein (MBP), expressed and biotinylated as described earlier (Figure 2B), was passed over an avidin-functionalized sensor chip. Its instantaneous interaction with the sensor chip was evident, as shown by a rapid increase in the SPR signal (yellow solid curve in Figure 2A). Subsequent washes with PBS did not remove any bound proteins from the chip surface, indicating a stable immobilization of the biotinylated protein to the avidin surface. To test the real-time interaction of MBP with its binding protein, anti-MBP antibody was flown over the sensor chip: a strong increase in the SPR signal (RU $\approx$ 5000) was observed (blue solid curve in Figure 2A), indicating specific binding of the antibody to MBP. The dissociation constant (K_d) of MBP/anti-MBP binding was estimated from the binding curve to be in 10^{-10}–10^{-11} M range. A 10 mM HCl solution was subsequently flown over the sensor chip, resulting in the regeneration of the sensor chip while retaining most of the biotinylated MBP on the surface. The slight decrease in the MBP signal (cyan dash line in Figure 2A) as a result of HCl treatments indicated that some immobilized MBP might have been washed off during the regeneration process. Second-round application of anti-MBP to the regenerated surface again resulted in an increase in SPR signal, albeit with ~50% of the first-round increase (pink solid curve in Figure 2A). Further rounds of regeneration/anti-MBP binding did not appreciably decrease the MBP signal, as well as that from anti-MBP binding (data not shown), indicating the initial decrease in MBP signal was probably due to dissociation of loosely associated MBP to monomeric avidin subunits on the original sensor chip. Once the amount of avidin-bound MBP on the surface was stabilized, further washes of the sensor chip were tolerated. This result is in good agreement with our previous findings that biotinylated proteins immobilized on an avidin-functionalized glass slide were able to withstand extremely harsh washing conditions.[22]

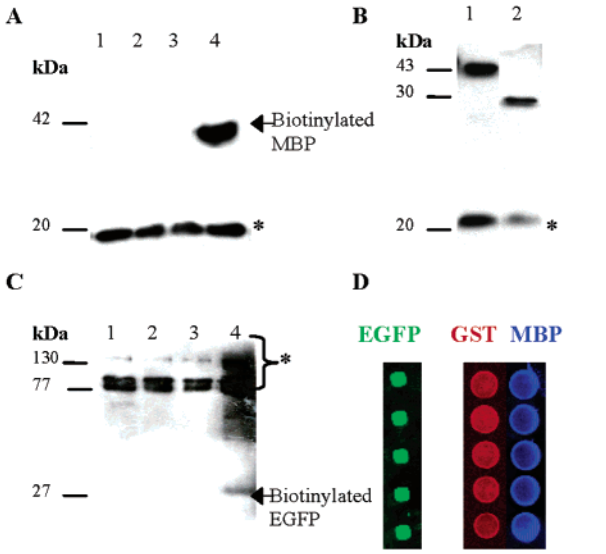

Figure 3. In vivo biotinylation of proteins and subsequent protein microarray applications. (A) In vivo biotinylation of MBP in *E. coli.* shown by western blots with anti-biotin. Lane 1: lysate of uninduced bacterial culture; lane 2: lysate of IPTG induced bacterial culture; lane 3: lysate of bacterial culture incubated with MESNA only; lane 4: lysate of bacterial culture incubated with MESNA and cysteine-biotin. See images of western blots with anti-MBP antibody in the Supporting Information. (B) In vivo biotinylation of yeast proteins (lane 1: YAL012W; lane 2: YGR152C) shown by western blots. Only biotinylated cell lysates were shown. The 20 kDa protein bands (*) in (A) and (B) correspond to acetyl-CoA carboxylase—the only biotinylated protein present in *E. coli.* (C) In vivo biotinylation of EGFP in mammalian cells shown by western blots. Lane 1: lysate of untransfected cells; lane 2: lysate of transfected cells; lane 3: lysate of transfected cells incubated with MESNA only; lane 4: lysate of transfected cells incubated with MESNA & cysteine-biotin. The three endogenous biotinylated mammalian proteins (*) were identified to be pyruvate carboxylase, methylcrotonyl CoA carboxylase and propionyl CoA carboxylase (from top to bottom). See images of western blots with anti-EGFP antibody in the Supporting Information. (D) Site-specific immobilization of biotinylated proteins onto avidin slides using bacterial crude lysates. Native fluorescence signal of EGFP (green) was observed whereas GST and MBP were individually detected with FITC-anti-GST (red) and Cy5-anti-MBP (blue), respectively.

In Vivo Biotinylation of Proteins. We next extended our intein-mediated biotinylation strategy to living cells. Although intein-mediated protein splicing is part of the naturally occurring processes in cells, its utilities in protein engineering have thus far been limited to in vitro applications.[24] The only exceptions are in the engineering of circulated proteins, where head-to-tail native chemical ligation occurred intramolecularly within live cells.[26] A recent report by Giriat et al. indicated that intein-mediated protein semi-synthesis was also possible in live cells between two designer protein fragments.[27] We reasoned that, if our cysteine-biotin tag is sufficiently cell-permeable, it may be able to cross the membrane of cells overexpressing a desired protein-intein fusion, cleave the fusion and at the same time biotinylate the target protein (method B in Scheme 1). We first tested the in vivo biotinylation of proteins in bacterial cells (Figure 3A,B). It was found that, following IPTG induction to overexpress the intein-fused protein in the growing bacterial cells, the addition of cysteine-biotin/MESNA to the growth media followed by further incubation of the cells resulted in a substantial level of biotinylation in the target protein. Modifica-

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tions of the cell growth, as well as the in vivo biotinylation conditions, further increased the level of protein biotinylation in the bacterial cells: up to an estimated 20−40% of all MBP expressed was observed to be biotinylated based on streptavidin absorption experiments (the Supporting Information). Biotinylation was observed in the target protein ONLY if both cysteine-biotin and MESNA were concomitantly added to the cell media (lane 4 in Figure 3A and the Supporting Information). We also showed that proteins from different biological sources (i.e., MBP and the two yeast proteins shown in Figure 3A,B respectively) could be efficiently biotinylated in live bacterial cells. The purity of the in vivo biotinylated proteins was confirmed by first incubating crude cell lysates with paramagnetic streptavidin beads, then analyzing the bead-bound proteins by SDS-PAGE and western blots. In all cases, the desired biotinylated protein could be isolated with high purity. The only impurity detected was acetyl-CoA carboxylase—an endogenous biotinylated protein known in *E. coli*. (Figure 3A,B).

We next tested thebiotinylation strategy in mammalian cells (Figure 3C). A mammalian expression vector was constructed such that it contains an EGFP gene fused to the intein. Transient transfection of the construct into HEK 293 cells resulted in an overexpression of green fluorescent proteins inside the cell, which could be readily followed by an UV lamp. Addition of cysteine-biotin/MESNA in basal media containing the transfected cells resulted in appearance of a new biotinylated protein band (Figure 3C, $M_w \approx 27$ KDa), corresponding to the apparent molecular weight of biotinylated EGFP. In addition, only three other biotinylated proteins were detected, which were also present in untreated cells, and they were identified to be the known naturally biotinylated proteins: pyruvate carboxylase, methylcrotonyl CoA carboxylase and propionyl CoA carboxylase. As shown in Figure 3C (e.g., lane 4), the expression of naturally biotinylated proteins appeared to be enhanced with the addition cysteine-biotin. This is probably caused by artifacts in our western blots due to extremely low protein expression level inherent to transient transfection experiments, although we could not completely rule out the possibility that the expression level of endogenous biotinylated proteins was enhanced with the addition of cysteine-biotin/MESNA. Further experiments need to be conducted to verify this observation. Attempts were also made to quantitate the amounts of uncleaved EGFP-intein fusion, the self-cleaved, as well as the biotinylated EGFP by western blots with anti-EGFP and anti-biotin antibodies, and it was found that majority of the expressed proteins in the mammalian cell lysates were the intein fusion and the self-cleaved product: only a small percentage (∼10%) of proteins expressed were biotinylated (see the Supporting Information). Work is currently underway to improve the in vivo protein biotinylation efficiency.

Taken together, these data demonstrated that, for the first time, the intein-mediated protein biotinylation worked inside different live cells (i.e., bacteria and mammalians). Compared to the in vitro method (i.e., method A in Scheme 1; see also the Supporting Information), the intein-mediated, in vivo protein biotinylation strategy presented herein is less efficient, requiring considerable refinements before it becomes useful for high-throughput proteomic applications. Nevertheless, it provides several obvious advantages: (1) pre-mature cleavage of the fusion protein in vivo may be alleviated by addition of cysteine-biotin/MESNA to growing cells, thus potentially maximizing the yield of the biotinlyated protein obtained; (2) excess biotin tag may be introduced during in vivo biotinylation, and readily removed at the end by simple washes of the cells; (3) following simple harvest and lysis of the cells, crude lysates containing the desired biotinylated proteins (together with other endogenous cellular proteins) may be used, without further purifications, for subsequent immobilization and downstream applications, i.e., one could simply spot crude lysates onto an avidin-coated glass slide to generate a protein array. Nonbiotinylated proteins in the cell lysate could be washed away on-chip in an efficient (e.g., protein immobilization and purification are done in a single step) and highly parallel (e.g., thousands of different protein spots could be processed simultaneously on a single glass slide) fashion, resulting in purified proteins immobilized on the microarray (vide infra). This is true because of the rare occurrence of naturally biotinylated proteins in the cell, and the highly specific and strong nature of biotin/avidin interaction, which can withstand extremely stringent washing/purification conditions otherwise impossible with other affinity tags.

Protein Microarray Generation. Next, we examined whether in vivo biotinylated proteins in the crude cell lysate could be used directly for protein microarray applications. We first biotinylated in vivo, as described above, three model proteins (EGFP, GST, and MBP). Following cell harvest and lysis, the crude lysates were spotted directly onto avidin-functionalized glass slides, washed and detected either by their native fluorescence (for EGFP) or with FITC-anti-GST and Cy5-anti-MBP, respectively (Figure 3D): native fluorescence of EGFP and specific bindings between the biotinylated proteins and their corresponding antibodies were observed. Nonbiotinylated proteins was not detected on the microarray (see the Materials and Methods), thus validating the binding specificity of biotinylated proteins to the avidin slide and suggesting the possible elimination of extra purification steps prior to spotting on a protein microarray. It should be pointed out that, one of the major challenges in protein array technologies is the ability of retaining the functional activity of proteins immobilized on the glass surface. In our experiments, the native fluorescence of the immobilized EGFP could be retained on the glass slide for weeks if stored properly at 4 °C (data not shown). Similar results were observed with protein arrays generated using proteins biotinylated in vitro,[22] highlighting the potential of our biotinylation strategies in protein microarray generation.

Protein Biotinylation in a Cell-Free System. We have thus far successfully demonstrated the utilities of intein-mediated biotinylation strategies in both in vitro and in vivo settings. In both cases, intein-fused proteins need to be successfully expressed in soluble forms in the host cell before biotinylation (either in vitro or in vivo) could take place. However, numerous problems may arise during protein expression in a host cell. The formation of inclusion bodies is one. This is especially true when one attempts to express eukaryotic proteins in prokaryotic hosts. Other problems include potential proteolytic degradation of the protein by endogenous proteases, as well as expression of proteins toxic to the host cell. Cell-free protein synthesis provides an attractive alternative for protein expression which may potentially overcome many of these problems (method C, Scheme 1), and is well-suited for protein microarray applications because (1) minute quantities of proteins generated in cell-free

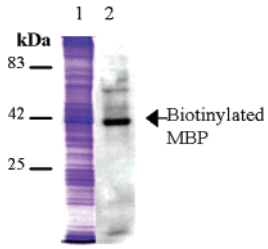

Figure 4. Protein biotinylation in a cell-free system. MBP fusion encoded by pTYB1-MBP-intein was first synthesized using the RTS cell-free system, followed by incubation with MESNA and cysteine-biotin. Proteins were precipitated and analyzed by a 12% SDS-PAGE gel. lane 1: coomassie stained image of the reaction; lane: western blots of lane 1 with anti-biotin antibody.

system are sufficient for spotting in a protein array, and (2) the method could be easily adopted in 96- and 384-well formats with a conventional PCR machine for potential high-throughput protein synthesis.[28] To assess whether our intein-mediated strategy is suitable for biotinylation of proteins expressed in a cell-free system, the MBP plasmid, containing MBP-intein fusion under the transcription control of T7 promoter, was used as the DNA template in a Rapid Translation System (RTS) 100 *E. coli*. HY kit. After cell-free protein synthesis, the reaction was incubated with cysteine-biotin/MESNA, followed by analysis with SDS-PAGE and western blots (Figure 4). The presence of a 42 kDa band on the anti-biotin immunoblot, and not any other band (Figure 4, lane 2), indicated successful and exclusive biotinylation of the MBP protein synthesized in the cell-free system. It should be noted that, among three protein biotinylation strategies presented herein (e.g., Scheme 1), the cell-free method seems to be the simplest of all. In our hands, however, it is also the least reliable: the efficiency of protein expression as well as the subsequent protein biotinylation depends greatly on a number of different factors, including the nature of the protein itself, the amount and quality of the DNA template used and the kind of cell lysates used for protein expression, etc. Experiments are currently underway to further assess some of these issues and results will be reported in due course.

Conclusions

Our intein-mediated biotinylation strategies have several advantages over other traditional methods in which biotin ligase is used. First, the precise splicing mechanism of intein allows coupling of biotin moiety to the C-terminus of proteins without introduction of additional amino acids sequences that otherwise may compromise the native protein activity. Second, most commonly used biochemical reagents do not inhibit the intein-mediated ligation reaction, thus enabling purification/biotinylation of the desired protein to be done efficiently in a single step. Third, cell toxicity due to overexpression of fusion proteins (unless the target protein is toxic to the host strain itself) is unlikely, because there is no competition of endogenous biotin consumption. Finally, as protein biotinylation is solely dependent on the cleavage of the fusion protein from the intein tag, use of expensive enzyme is not required and coexpression of biotin ligase is not necessary for in vivo biotinylation of proteins.

Our findings herein indicate that the intein-mediated biotinylation approaches are sufficiently general and versatile, enabling proteins from different biological sources to be site-specifically biotinylated under different conditions, and subsequently used in a wide range of avidin/biotin technologies. Expressed proteins fused to an intein tag could be efficiently purified and biotinlyated, in vitro, in a single purification step. We also showed that the strategy works in both live bacterial and mammalian cells. We further showed intein-fused proteins synthesized from a cell-free system could undergo intein-mediated biotinylation reaction as well. We emphasized the unique utilities of our strategies in the area of protein microarray, as this technology may be one of the most powerful tools for high-throughput analysis of protein functions. Several essential aspects of a protein microarray were addressed using our intein-mediated biotinylation strategies. First, protein biotinylation/immobilization was site-specific, leading to uniform orientation, and more importantly retention of the functional integrity of proteins immobilized on the array. Second, no extra macromolecular tag was introduced in the immobilized protein, further ensuring the biological activity of proteins was minimally perturbed. Third, avidin/biotin interaction was extremely stable, enabling immobilized proteins to be thoroughly washed to remove cellular contaminants, and subsequently screened under even the most stringent conditions. Finally, the strategy, upon modifications, does not involve tedious protein purification/elution steps and allows the facile generation of biotinylated proteins (e.g., in live cells or in a cell-free system), making it possible for proteins in crude lysates to be spotted directly onto a protein array. This enables expression, without further processing (e.g., purification and elution, etc.), of a large array of biotinylated, ready-to-spot proteins in a truly high-throughput, high-content fashion.

Key challenges remain with the strategies presented herein, and none is more pressing than to further improve the efficiency of protein biotinylation in live cells. We are also exploring the feasibility of incorporating a variety of cysteine-containing molecular probes at the C-terminus of proteins (e.g., fluorescent and photocrossing probes) for various biochemical and biophysical studies of proteins, both in vitro and in vivo.[29,30] New findings will be reported in due course.

Abbreviations: ORF, open reading frame; EGFP, enhanced green fluorescent protein; MBP, maltose binding protein; PCR, polymerase chain reaction; SPR, surface plasmon resonance; MESNA, 2-mercaptoenthanesulfonic acid; AA, amino acid; GST, glutathione-*S*-transferase.

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Supporting Information Available: SDS-PAGE gels of data summarized in Table 1, streptavidin absorption experiments, as well as experiments to assess in vivo biotinylation efficiency in bacterial and mammalian cells (4 pages). This material is free of charge via the Internet at http://pubs.acs.org.

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