DNA-Templated Chemistry

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Photoaffinity Labeling of Small-Molecule-Binding Proteins by DNA-Templated Chemistry**

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Dedicated to the Bayer company on the occasion of its 150th anniversary

Small molecules are important tools in exploring biology.[1] Accurate characterization of small-molecule- (SM) binding proteins is of great importance in applications such as target identification and proteomic profiling,[2] which calls for general, specific, and high-throughput methods to characterize the multitude of SM–protein interactions in the proteome.

Classic "affinity pull-down" employs immobilized or tagged SM probes to isolate binding proteins (Figure 1a, left and center); but they are usually limited to high abundance/affinity SM–protein interactions. Covalent affinity probes, equipped with additional reactive groups, provide a straightforward way for protein capture and isolation (Figure 1a, right). Since first developed in 1994,[3] these probes received extensive use in numerous studies.[4] However, the basic probe architecture with multiple functions combined in one entity remains largely unchanged, which lacks modularity and presents several issues. First, the crosslinking group should be distal from the binding site in order not to alter binding, but it also should be close to the protein for efficient crosslinking. Recently, Cravatt and coworkers incorporated benzophenone, a crosslinking group, as part of the binding structure. This elegant strategy solves the dilemma, but probes are then limited to benzophenone derivatives.[4f] Second, for each SM, preparation of multiple probes for different purposes are often required (e.g. with a fluorophore for detection and with an affinity tag for purification). Finally, probes often face proteins with varied binding properties. A collection of probes with diverse small molecules is highly desired for proteome-wide protein profiling.[5] Previously, most affinity probes offer very limited multiplexing capability.

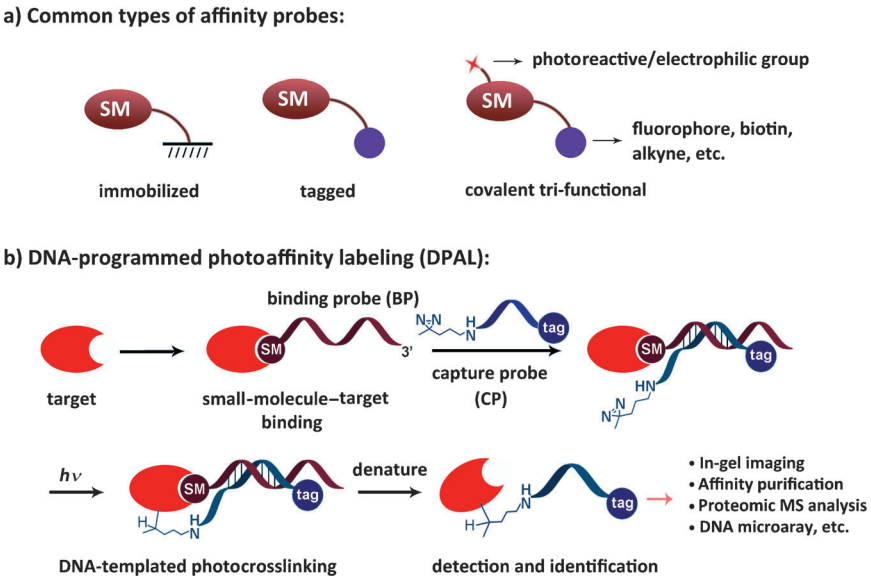

Figure 1. a) Common types of affinity probes. b) Scheme of DNA-programmed photoaffinity labeling (DPAL).

Here we report a novel method named "DNA-programmed photoaffinity labeling (DPAL)". DNA-templated chemistry has emerged as a versatile approach to control chemical reactions;[6] and DNA was used for protein detection in immuno-polymerase chain reaction (immuno-PCR)[7] and proximity-ligation assay.[8] Recently nucleic acids (DNA and PNA) have been used to program the assembly of SM ligands with controlled combinations or distance to explore spatial information of ligand binding to protein targets.[9] We implemented DNA-templated chemistry to establish a simple, modular, and multiplexed system for labeling the binding proteins of small molecules.

DPAL consists a dual-probe system (Figure 1b): the small molecule is conjugated to a DNA as the "binding probe (BP, shown in red)"; and a photocrosslinking group is conjugated

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to a complementary DNA as the "capture probe (CP, shown in blue)". After BP/CP hybridization, irradiation triggers DNA-templated photocrosslinking of CP to covalently capture the protein. DPAL decouples SM binding from crosslinking/tagging. The crosslinking group can be flexibly adjusted by DNA hybridization. In DPAL, only a single modification (for DNA conjugation) on the SM is needed and only a single BP is necessary, as BP can be paired with multiple CPs for different purposes. Importantly, the introduction of DNA provides multiplexing capability.

We first addressed an important issue in affinity labeling: nonspecific crosslinking, a particularly severe issue for labeling low-abundance/low-affinity proteins when a high probe concentration is necessary.[10] It is well known that the salt concentration can affect specific and nonspecific molecular interactions, such as protein–DNA,[11] protein–protein,[12] protein–ligand,[13] and protein–matrix interactions.[14] Interestingly, this phenomenon has not been widely exploited to reduce nonspecific labeling by affinity probes. To test this possibility, we first investigated photocrosslinking reactions between a diazirine/fluorescein-labeled DNA probe (DZ/FAM-CP, Figure 2) and bovine serum albumin (BSA), a protein well-known for its extensive nonspecific interactions with small molecules.[15] Significant BSA photocrosslinking by DZ/FAM-CP was observed at concentrations $>5\ \mu M$ in H_2O (Figure 2a, top); in sharp contrast, with additional salt ($1\times$ PBS + 0.1 M NaCl), crosslinking was nearly completely suppressed even with 100 μM DZ/FAM-CP (Figure 2a, bottom). In HeLa cell lysate (alone or with purified BSA), crosslinking was also suppressed by the added salt (Figure 2b, lanes 2 and 4), indicating this effect is not limited to BSA but rather general to other cellular proteins. We also performed studies regarding protein and salt concentrations, salt type, irradiation time, and other types of probes. The results collectively suggest the salt effect is quite general. The ionic strength appears to be the only major effecting factor (see the Supporting Information for details). This salt effect has been implemented throughout experiments in this study.

Next, we examined whether specific SM–target interactions can be identified by DPAL under elevated salt concentrations. First, we conjugated a small molecule LPCBS (**1**) to the 5′-NH_2 of a 27 bp DNA as the binding probe (LPCBS-BP) to label its well-known target carbonic anhydrase II (CA-II, purified, 10 μM, $K_d = 0.9$ nM for free **1**)[16] with BSA (10 μM) as the background. DNA conjugation at the C-terminus of LPCBS retains its binding property.[17] A 15 bp DZ/FAM-CP fully complementary to LPCBS-BP was prepared as the CP. After BP binding, CP hybridization, and irradiation, a 24 bp displacement DNA complementary to BP was added to eliminate the possibility of forming a noncovalent complex of protein-bound BP/CP duplex. As shown in Figure 3a, SDS-PAGE results show DZ/FAM-CP labeled CA-II only in the presence of complementary BP/CP after irradiation (Figure 3a, left panel, lanes 1–2). Even with excess probes (20 μM), nonspecific BSA labeling was not detected. All negative controls (no irradiation, no BP, mismatched BP/CP, BP without LPCBS, no CA-II, denatured CA-II; Figure 3a, lanes 3–8) gave no detectable CA-II or BSA labeling, indicating the labeling requires specific SM–protein binding and DNA hybridization. As expected, without added salt, significant BSA labeling was observed (see Figure S2 in the Supporting Information). Next, we mixed purified CA-II with Jurkat cell lysate and performed similar DPAL experiments with same probes, again specific labeling was observed despite the presence of numerous cellular proteins and excess probes (Figure 3a, center and right panels). Encouraged by this result, we performed DPAL experiments on a series of SM–protein pairs covering a wide range of binding affinities (structures shown in Figure 3b)[17] with the same DPAL experiment setup (purified proteins in a BSA background); again similar labeling performances were observed (Figure 3c). In addition, incubation with DNase I diminished the FAM signal (lane 9), further confirming the covalent labeling by DPAL probes.

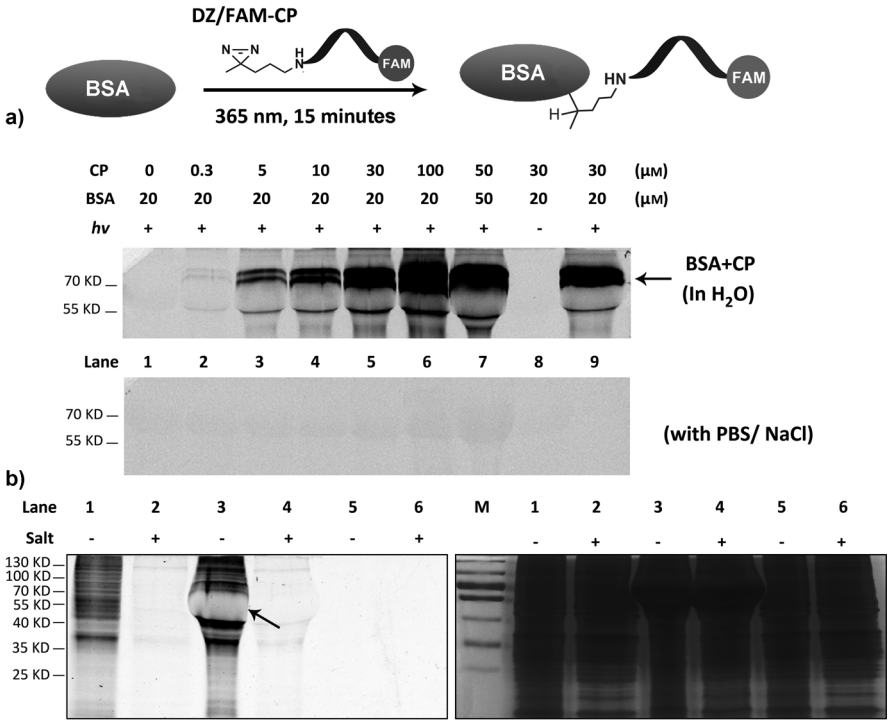

Figure 2. Suppression of nonspecific crosslinking by elevated salt concentrations. a) Crosslinking between BSA and DZ/FAM-CP in H_2O and in $1\times$PBS/0.1 M NaCl, monitored by FAM. Lanes 1–7: with irradiation (concentrations are as marked). Lane 8: no irradiation. Lane 9: PBS added after irradiation in H_2O. b) Photocrosslinking between BSA and DZ/FAM-CP in lysate (HeLa, 1.0 mg mL^{-1},10 μg per lane; CP: 20 μM). Left: fluorescence. Right: silver stain. M: marker. Lanes 1 and 2: lysate only. Lanes 3 and 4: lysate + 20 μM BSA. Lanes 5 and 6: lysate without irradiation. The arrow indicates the position of BSA-CP conjugate.

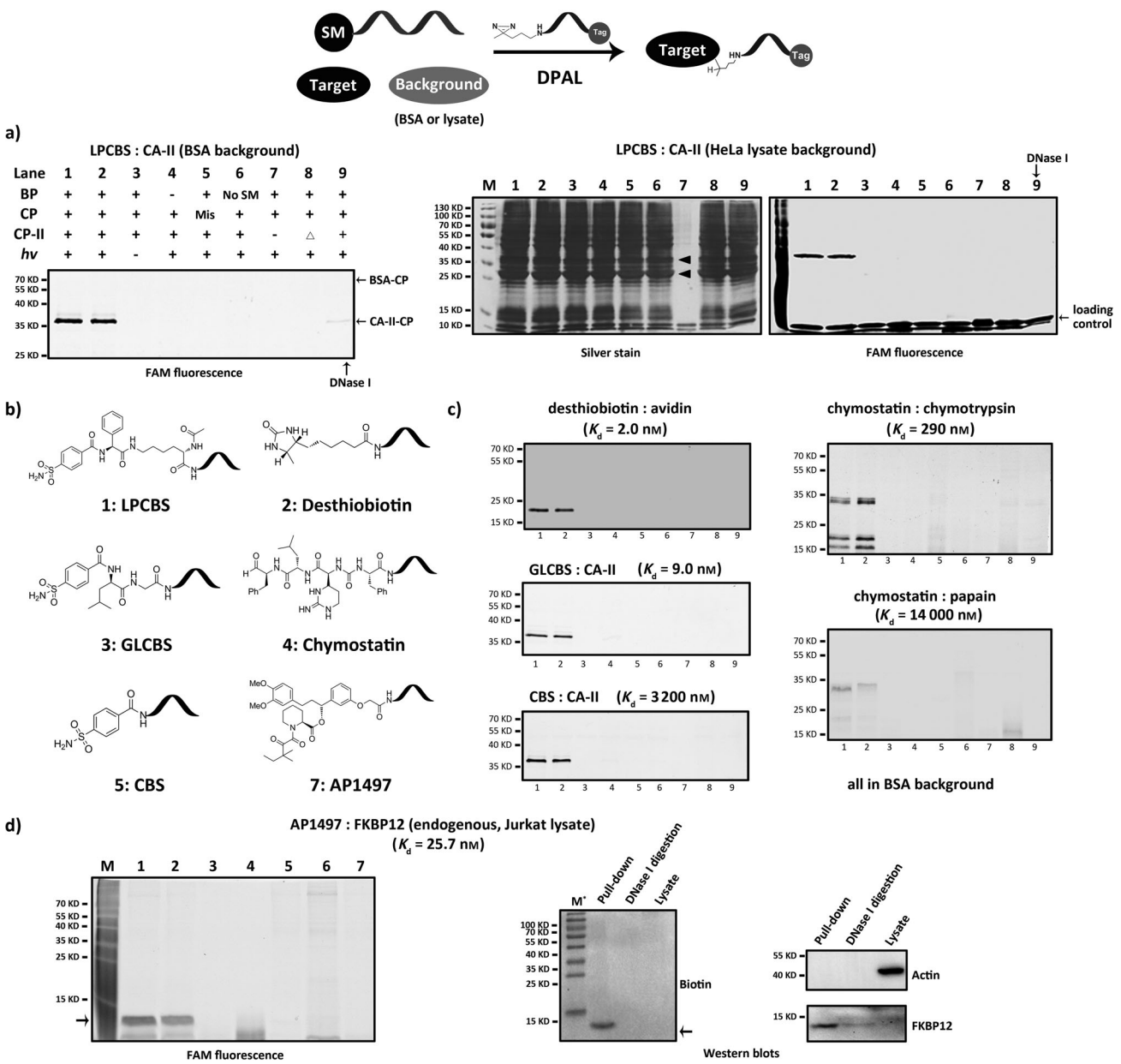

Figure 3. a) DPAL of CA-II by LPCBS (**1**)-BP in BSA background (left) and in HeLa cell lysate (middle & right). CA-II, BSA: 10 μM. BP/CP: 20 μM. Lysate: 10 μg/lane. Lane 1: BP/CP/*hν*; lane 2: pre-hybridized BP/CP/*hν*; lane 3: no *hν*; lane 4: no BP; lane 5: mismatched CP; lane 6: BP without SM; lane 7: no CA-II or lysate; lane 8: CA-II pre-denatured; lane 9: with DNase I. Arrows indicate positions of CA-II and CA-II/CP conjugate. Loading control: a 50 bp, FAM-labeled DNA. b) Structures of SMs used (**1**, LPCBS; **2**, desthiobiotin; **3**, GLCBS; **4**, chymostatin; **5**, CBS; **7**, AP1497). c) DPAL results of **2**–**5** with respective targets. Conditions and lane sequences are the same as in (a). d) DPAL of AP1497s endogenous target FKBP12 in Jurkat cell lysate. Left panel: fluorescent image with DZ/FAM-CP. The lane sequence is the same as in (a). Jurkat lysate: 3.6 mg mL^{-1}, 54 μg per lane. Middle and right panels: after biotin-CP labeling, streptavidin beads enrichment, eluted proteins are detected by anti-biotin and FKBP12 antibodies. M: marker. "→": expected positions of FKBP12. M*: overlaid marker.

Next, we applied DPAL in labeling an endogenous protein in cell lysate by using AP1497 (**7**), an immunophilin inhibitor with a well-known target FKBP12.[18] In Jurkat cell lysate, AP1497-BP (Figure 3b), along with DZ/FAM-CP, specifically labeled a protein matching the molecular weight of FKBP12 (Figure 3d, left). Furthermore, taking advantage of the modularity of DPAL, without preparing additional BP, we simply used a 5′-biotin-, 3′-diazirine-labeled DNA as CP (biotin-CP) to pair with the existing BP. After DPAL and filtration to remove free probes (ultracentrifugal tube, MWCO: 30 kDa), streptavidin beads pull-down and elution enriched a single protein band, which can be blotted by biotin- and FKBP12-specific antibodies (Figure 3d, middle and right panels), proving the identity of FKBP12 labeled by biotin-CP.

We intentionally used high protein concentrations (10 μM) and excess probes (20 μM) to challenge DPAL, for which labeling is both SM-specific and DNA sequence-specific. This

has been demonstrated by the absence of protein labeling, either specific or nonspecific, in negative controls. For example, experiments with the mismatched BP/CP or without SM did not give detectable target or BSA labeling even with excess probes.

It is common in affinity labeling that only a low-affinity probe is available and target proteins are not abundant; therefore we explored the performance of DPAL in labeling low-abundance/low-affinity proteins. We spiked HeLa cell lysate with purified CA-II at various dilutions to mimic the low-abundance target scenario. Again, we simply used the biotin-CP to pair with existing GLCBS-BP and CBS-BP (Figure 3b, **3** and **5**). Free CBS has a K_d of 3.2 μM against CA-II,[19] which can be considered as a low-affinity ligand. After DPAL and filtration to remove free probes, we performed streptavidin pull-down to enrich any biotin-CP-labeled proteins from the mixture. Figure 4 shows both probes specifically enriched a protein matching the expected molecular weight of CA-II (lane 4; anti-biotin Western blot), even with the low-affinity CBS probe at 1:5000 dilution (Figure 4c; see Figure S10 for explanation of the two bands). With free GLCBS, no enriched protein bands were observed (lane 5), indicating the labeling is specific for small molecules. With cell lysate only, no CA-II band was observed, proving the enrichment was not from endogenous expression (lane 6). Carefully optimized tri-functional probes can label less than 0.1 wt% of proteins in cell lysate.[20] Our data show DPAL is comparable to conventional probes (Figure S11), although both approaches benefited significantly from the salt effect to suppress nonspecific labeling in this study. Nevertheless, the two-probe system of DPAL provides simple probe preparation and novel modularity in protein labeling and enrichment.

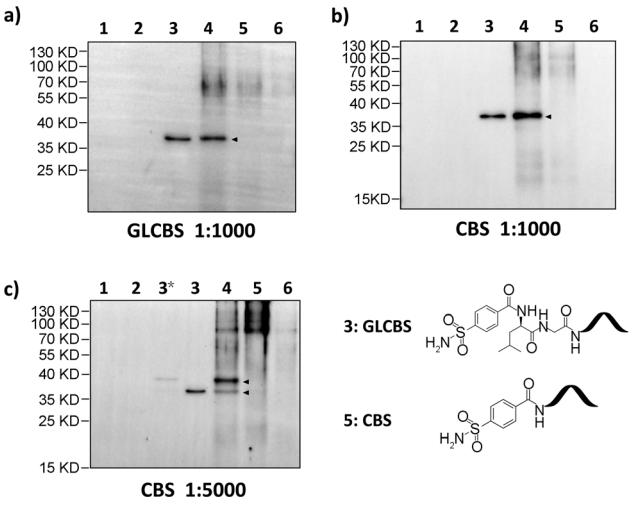

Figure 4. Biotin Western blots of enriched CA-II from HeLa lysate with a) GLCBS-BP (CA-II:lysate 1:1000), b) CBS-BP (CA-II:lysate 1:1000), and c) CBS-BP (CA-II:lysate 1:5000). Lane 1: lysate only; lane 2: CA-II only; lane 3: CA-II + CP in H_2O with $h\nu$; lane 3*: CA-II + CP/BP in PBS/NaCl with $h\nu$; lane 4: elution after enrichment; lane 5: same as 4 but with GLCBS competition; lane 6: same as 4 but with lysate only (no CA-II added). BP, CP: 20 μM. Ratios: w:w. See the Supporting Information for enrichment procedures. Dark smears in gels are due to formamide used in elution buffer.

In DPAL, we can flexibly shift the hybridization position of CP relative to the protein target. We selected four SM–target pairs (Figure 3b, **2**, **3**, **4**, **5**) and prepared multiple CPs with varied "*n* values", able to hybridize at different locations on these BPs (Figure 5). We performed DPAL experiments with these SM–target pairs (with purified proteins) and compared their labeling yields. Results show that DNA-templated photocrosslinking is "distance-dependent":[21] in general, CPs with negative *n* values have higher yields than positive *n* values. Interestingly, $n = -3$ appears to be optimal, instead of the typical "side-by-side" alignment in most DNA-templated reactions. Overall, these data suggest the flexible DNA hybridization can be used to tune for optimal cross-linking in DPAL.

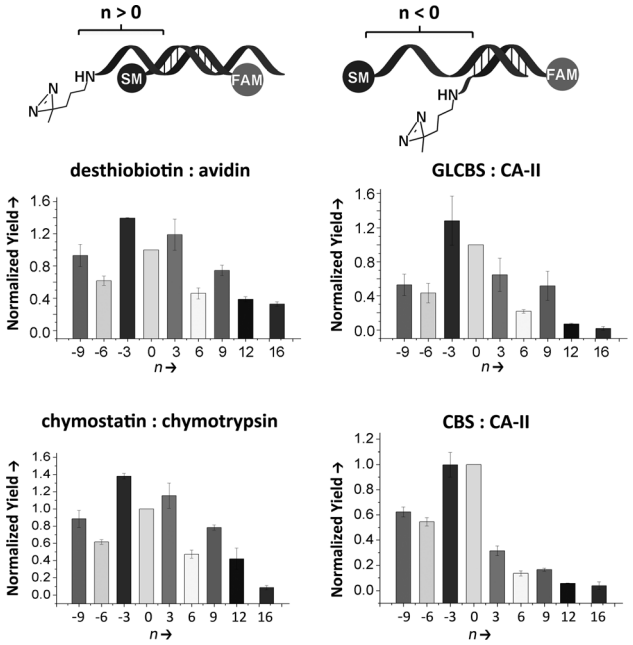

Figure 5. Effect of the CP hybridization position (different *n* values) on labeling yields with four SM–target pairs. SMs and their targets are labeled above each plot. DPAL conditions are the same as in Figure 3a. Labeling yields were determined by an internal FAM-labeled 50 bp DNA and normalized to $n = 0$. Error bars (standard deviation, SD) are based on three replicates of each experiment.

Conventional affinity probes have very limited multiplexing capability. Previously, probes with two-color fluorescent tags[22] and chemically orthogonal handles have been reported.[4e] Activity-based protein profiling (ABPP) has been accomplished with PNA-based probes.[23] To demonstrate the multiplexing capability of DPAL, we mixed avidin (i), CA-II (ii), and trypsin (iii) at 1:1:1 molar ratio along with HeLa cell lysate as the background. These proteins have different molecular weights so that formed protein–DNA conjugates can be easily distinguished by gel mobility (Figure 6a). The mixture was subjected to DPAL with different combination of BPs (structures shown in Figure 6a) and complementary DZ/FAM-CPs. We supplemented mis-

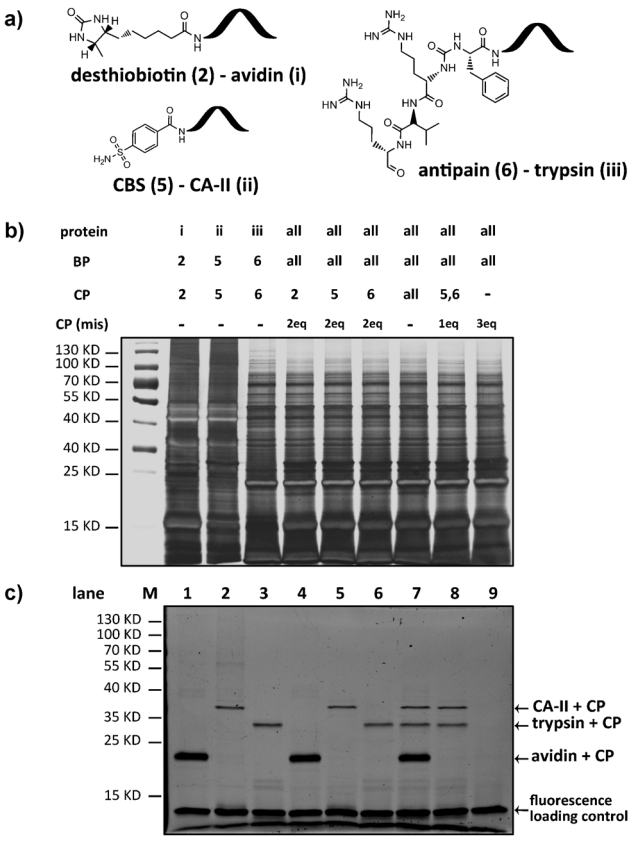

Figure 6. SDS-PAGE analysis of multiplexed DPAL. a) Structure of BPs and known targets; b) silver stain showing complete proteins in the mixture; c) FAM fluorescence showing proteins specifically labeled by DZ/FAM-CP. Conditions: proteins, 10 μM; BP and CP, 20 μM; lysate: 1.0 mg mL^{-1}, 10 μg per lane. M: marker. Lanes 1–3: controls with individual BP/CP pair and respective protein target; lanes 4–6: all three proteins and BPs with only a single complementary CP; lane 7: all three proteins, BPs, and CPs; lane 8: all three proteins and BPs with two complementary CPs; lane 9: same as 7 with only mismatched CP. CP(mis): mismatched CP.

matched CPs when necessary so that the overall CP concentration remains constant in all experiments. As shown in the figure, despite the presence of cellular proteins and other non-target proteins (Figure 6b), DZ/FAM-CP selectively labeled the respective targets of the SM depending on the sequence complementarity of added CP strands, either individually (lanes 4–6, with only one complementary CP) or in combinations (lanes 7–8, with 3 or 2 complementary CPs) without probe cross-reactivity (Figure 6c). Experiments with mismatched CP did not show any protein labeling on either target or cellular proteins (lane 9). This result demonstrated the multiplexing capability of DPAL in affinity labeling with multiple small molecule probes in a single solution.

In summary, we have developed a novel affinity labeling method based on DNA-templated photocrosslinking chemistry. DPAL uses a modular system to dissect binding from other functions that are usually combined in one probe, therefore simplifying probe design and preparation. Structure–activity relationship information on acceptable modification site on the SM is still required as any affinity probes; however, only a single DNA conjugation and a single binding probe are needed in DPAL. Capture probes are universal, and can actually be pre-prepared and stored as a kit, ready to pair with any BP regardless of a specific small molecule used.

The introduction of DNA in DPAL provides not only simplicity and modularity, but also multiplexing capability lacking in most conventional affinity probes. We used a three-target mixture only as a model system to show that DNA provides DPAL with virtual spatial separation and probe encoding, two key features necessary for multiplexed affinity labeling. We expect a combination with DNA microarray would significantly improve the multiplexability and throughput of DPAL. Moreover, DPAL may also be used as a sensitive protein detection method benefiting from DNA amplification. Our laboratory is actively applying DPAL in research programs exploring these opportunities.

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