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TECHNICAL NOTE

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Purification of Tetracysteine-Tagged Proteins by Affinity Chromatography Using a Non-Fluorescent, Photochemically Stable Bisarsenical Affinity Ligand

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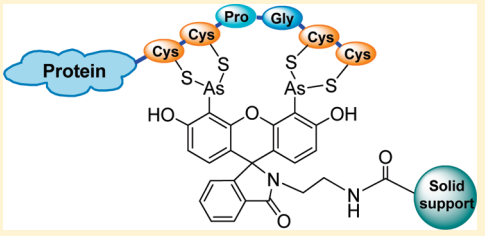
ABSTRACT: The use of genetically encoded small peptide tags such as polyhistidine and tetracysteine tags has become important for protein purification and enrichment. An improved affinity purification of tetracysteine (CCXXCC) tagged proteins has been achieved using a nonfluorescent, photochemically stable bisarsenical affinity ligand SplAsH. The photochemical stability of the SplAsH-biotin, shown in compound **5**, is superior to FlAsH-EDT$_2$ and ReAsH-EDT$_2$. An application of the SplAsH tag for affinity purification of tetracysteine-tagged proteins is reported.



INTRODUCTION

Protein modification with reporter tags has allowed the study of protein localization and protein−protein interactions inside living cells.[1−3] Traditionally, the protein of interest has to be isolated *in vitro*, labeled with a reporter tag, then reintroduced into cells by microinjection or electroporation. The use of genetically encoded fluorescent proteins such as green fluorescent protein (GFP) has provided a simple and powerful tool to study protein dynamics in cell biology.[4,5] However, GFP is a large protein (27 kDa), which could interfere with the physiological role of the protein of interest.[6,7] Small peptide tags, such as the hexahistidine (His$_6$) tag and tetracysteine (CCPGCC) tag, have greater advantage than GFP in protein labeling, because the small tags have minimal interference with the protein of interest.[6,8] The tetracysteine tag combined with either fluorescein arsenical hairpin binder (FlAsH-EDT$_2$, Figure 1) or Resorufin arsenical hairpin binder (ReAsH-EDT$_2$, Figure 1) provide useful tools for site-specific labeling of recombinant proteins inside live cells.[9−12] An especially important and useful feature of these molecules is their fluorogenicity; the small molecules FlAsH-EDT$_2$ and ReAsH-EDT$_2$ are membrane-permeant and almost nonfluorescent, so they can easily get into cells, then they bind with high affinity and specificity to the tetracysteine domain, causing the protein-bound dyes to become highly fluorescent.

On the basis of the high affinity and high specificity of the interaction of biarsenical dyes with the tetracysteine motif, methods using biarsenicals immobilized on a matrix as a stationary phase for affinity purification of tetracysteine-tagged proteins have been developed.[11,13,14] The interaction between biarsenical dyes and the tetracysteine motif can be easily reversed by incubation with dithiols such as 1,2-ethanedithiol (EDT) or dithiothreitol (DTT). This gives a gentle and mild elution condition with minimal disruption of protein structure and activity.

The most commonly used His$_6$-tag proteins are purified by binding to metal ions (Ni^{2+} or Co^{2+}) which are chelated to an affinity matrix followed by elution with high concentrations of imidazole or low pH.[15] This condition may disrupt some protein structure and activity.[16] In addition, the purified protein is often contaminated with intrinsic histidine-rich proteins and small amounts of metal ions, which may inhibit many enzyme activities.[11,13]

The major limitation to using the FlAsH affinity matrix for purification of tetracysteine-tagged proteins is a stability issue. The compounds FlAsH-EDT$_2$ and ReAsH-EDT$_2$ are photochemically unstable,[17] which limits their wide application in purification of tetracysteine-tagged proteins. We report herein on our successful efforts to apply a photochemically stable bisarsenical affinity ligand for affinity purification of tetracysteine-tagged proteins.

EXPERIMENTAL PROCEDURES

General Materials and Methods. All chemicals and solvents were purchased from Aldrich, Acros, or TCI and were used without further purification. Thin layer chromatography (TLC) was performed on aluminum-backed plates with silica gel 60 with F_{254} indicator. Flash column chromatography was carried out on silica gel (230−400 mesh). High-performance liquid chromatography (HPLC) analysis was performed on a Waters 600 instrument equipped with a Waters photodiode array detector. All moisture- or air-sensitive reactions were carried out under a static argon atmosphere. ^{1}H NMR (400 MHz) spectra were recorded at room temperature on a Bruker ADVANCE-400 spectrometer with residual proton resonances in deuterated solvents (DMSO-d_6, or MeOD-d_4) as the internal standard. LC/MS spectra were obtained with a Waters micromass ZQ instrument.

***tert*-Butyl 2-(3′,6′-dihydroxy-3-oxospiro[isoindoline-1,9′-xanthene]-2-yl) ethylcarbamate (1).** Fluorescein methyl ester was prepared according to a literature procedure from

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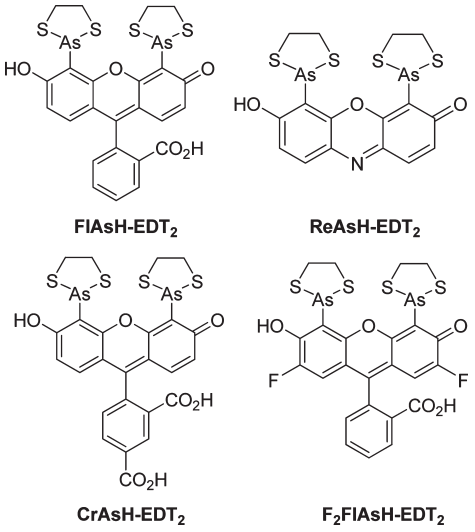


Figure 1. FlAsH-EDT$_2$, ReAsH-EDT$_2$, and related analogues.

fluorescein in 95% yield.[18] Fluorescein methyl ester (1.0 g, 2.9 mmol), and BocHNCH$_2$CH$_2$NH$_2$·HCl (1.14 g, 5.8 mmol) were dissolved in 10 mL DMF. Triethylamine (806 μL, 5.8 mmol) was added. The reaction mixture was heated at 100 °C overnight in a sealed pressure tube. After cooling to room temperature, the solvent was evaporated *in vacuo*. The residue was dissolved in ethyl acetate (80 mL); washed with H$_2$O (40 mL), 0.1 M HCl (40 mL), and brine (40 mL); dried over Na$_2$SO$_4$; and concentrated *in vacuo*. The crude mixture was purified by column chromatography on SiO$_2$ (EtOAc/Hexane = 1:1−2:1) to afford **1** (1.12 g, 2.4 mmol, 82% yield) as a white solid. The obtained compound matches with data that is in the literature.[17]

***tert*-Butyl 2-(4′,5′-bis(mercuric acetate)-3′,6′-dihydroxy-3-oxospiro[isoindoline-1,9′-xanthene]-2-yl) ethylcarbamate (2).** Compound **1** (1.1 g, 2.3 mmol) was dissolved in 60 mL ethanol, then the solution was added to a solution of mercuric acetate (1.53 g, 4.8 mmol) in 4 mL HOAc and 100 mL H$_2$O. The reaction mixture was stirred at 65 °C overnight in a sealed pressure tube. After cooling to room temperature, the resulting white precipitate was filtered through filter paper, washed with H$_2$O (3 × 20 mL), and then dried under high vacuum to afford **2** (2.05 g, 2.1 mmol, 90% yield) as a white solid. The obtained compound matches with data that is in the literature.[17]

***tert*-Butyl 2-(4′,5′-bis(1,3,2-dithiaarsolan-2-yl)-3′,6′-dihydroxy-3-oxospiro [isoindoline-1,9′-xanthene]-2-yl) ethylcarbamate (3).** Compound **2** (1.0 g, 1.0 mmol) was suspended in 20 mL THF. Di-isopropylethylamine (DIPEA, 1.4 mL, 8.0 mmol), AsCl$_3$ (1.6 mL, 20 mmol, caution: very toxic), and Pd(OAc)$_2$ (20 mg) were added. The reaction mixture was stirred at 50 °C for 2 h under argon, then at room temperature overnight under argon. The reaction mixture was poured into acetone/aqueous phosphate buffer (1:1, 0.5 M K$_2$HPO$_4$, pH 7, 100 mL) containing ethanedithiol (EDT, 3 mL). After stirring for 30 min, CHCl$_3$ (50 mL) was added, and the mixture was stirred for another 30 min. The organic layer was collected, and the aqueous layer was washed with CHCl$_3$ (3 × 30 mL). The combined organic layers were dried over Na$_2$SO$_4$ and concentrated *in vacuo*. The crude mixture was purified by column chromatography on SiO$_2$ (elute first with toluene to remove arsenic−EDT complex, then elute with EtOAc/Hexane = 2:3) to afford **3** (760 mg, 0.94 mmol, 94% yield) as a white solid. The obtained compound matches with data in the literature.[17]

2-(2-Aminoethyl)-4′,5′-di(1,3,2-dithiarsolan-2-yl)-3′,6′-dihydroxyspiro[isoindoline-1,9′-xanthen]-3-one (4). Compound **3** (300 mg, 0.37 mmol) was dissolved in 8 mL CH$_2$Cl$_2$ at 0 °C. TFA (4 mL) was added. The reaction mixture was stirred at 0 °C for 2 h. The reaction mixture was concentrated *in vacuo*. The residue was precipitated in diethyl ether and dried under reduced pressure via a vacuum pump to afford **4** (290 mg, 0.35 mmol, 95% yield) as a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.01 (s, 2H), 7.85 (dd, 1H, J = 2.0, 6.6 Hz), δ 7.60 (s, 2H), 7.56 (m, 2H), 7.05 (dd, 1H, J = 2.0, 6.6 Hz), 6.52 (m, 4H), 3.50 (m, 8H), 3.26 (t, 2H, J = 5.8 Hz), 2.39 (m, 2H). ESI-MS $[M+H]^+$ m/z calcd. for 706.91, found 706.87.

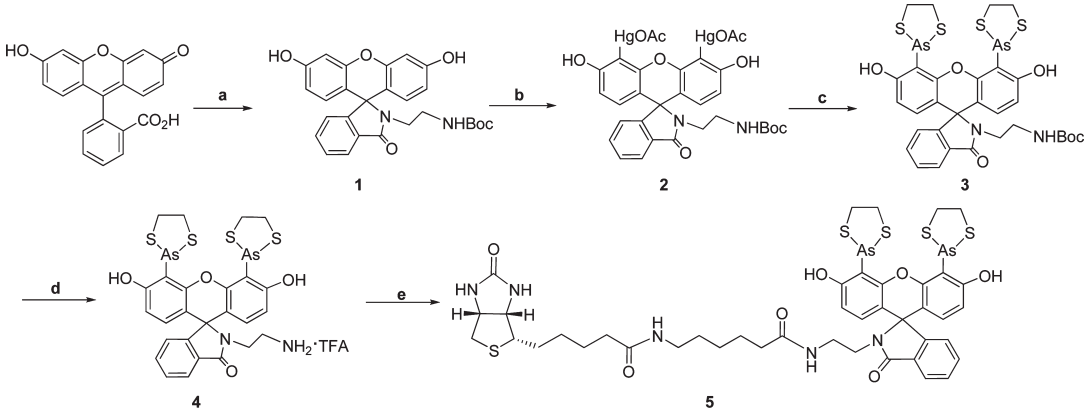
***N*-(2-(4′,5′-Di(1,3,2-dithiarsolan-2-yl)-3′,6′-dihydroxy-3-oxospiro[isoindoline-1,9′-xanthene]-2-yl)ethyl)-6-(5-((3a*S*,-4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)hexanamide (5).** Compound **4** (72 mg, 0.09 mmol) and 6-((biotinoyl)amino)hexanoic acid succinimidyl ester (40 mg, 0.09 mmol, Life Technologies) were dissolved in 3 mL dry DMF. TEA (37 μL, 0.26 mmol) was added. The reaction mixture was stirred at room temperature for 1 h. Diethyl ether (20 mL) was added to the reaction mixture. The precipitate was collected by centrifuge and purified by column chromatography on SiO$_2$ (CHCl$_3$/MeOH = 10:1) to afford **5** (80 mg, 0.08 mmol, 85% yield) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.95 (s, 2H), 7.90 (dd, 1H, J = 2.0, 6.6 Hz), 7.58 (m, 2H), 7.06 (dd, 1H, J = 2.0, 6.6 Hz), 6.50 (m, 4H), 4.50 (m, 1H), 4.30 (m, 1H), 3.60 (m, 8H), 3.18 (m, 5H), 2.99 (m, 3H), 2.71 (m, 1H), 2.19 (t, J = 7.2 Hz, 2H), 2.03 (t, J = 7.2 Hz, 2H), 1.66−1.30 (m, 12H). ESI-MS $[M+H]^+$ m/z calcd. for 1046.07, found 1046.34.

Synthesis of Affinity Matrix 6. CrAsH-EDT$_2$ succinimidyl ester was prepared according to a literature procedure.[11] To a 20 mL plastic, fritted column with a Teflon stopcock and a cap to seal the column, 10 mL of amino-agarose (Affi-Gel 102, Biorad) in PBS buffer containing 0.1 mM EDTA, 5 mM β-mercaptoethanol (BME), and 0.1 mM EDT, pH 7.4 was added, followed by addition of 1 mL of CrAsH-EDT$_2$ succinimidyl ester (10 mM solution in DMSO, 10 μmol) and 0.5 mL of 1 M NaHCO$_3$. The resin was mixed at RT for 4 h by end-to-end rotation, and washed extensively with the same buffer. Capping of free amines was achieved by treatment with a 10-fold excess of *N*-acetoxysuccinimide (prepared from acetic acid and *N*-hydroxysuccinimide with dicyclohexylcarbodiimide in CH$_2$Cl$_2$) over total amines (~12 μmol/mL) in the same phosphate buffer with slow rotation for additional 2 h. The immobilized affinity matrix **6** was washed and stored in 50% EtOH at 4 °C.

Synthesis of Affinity Matrix 7. To a 20 mL plastic, fritted column with a Teflon stopcock and a cap to seal the column, 10 mL of *N*-hydroxysuccinimidyl ester (NHS) activated agarose (Affi-Gel 10, Biorad) in 1/1 isopropanol/DMF was added, followed by addition of 1 mL of compound **4** (20 mM solution in DMSO, 20 μmol) and 0.2 mL of TEA. The resin was mixed at RT for 2 h by end-to-end rotation, and washed with isopropanol and DMF. Unreacted NHS groups were quenched by treatment with 0.5 M ethanolamine in isopropanol for an additional 1 h. The immobilized affinity matrix **7** was washed with PBS buffer containing 0.1 mM EDTA, 5 mM BME, and 0.1 mM EDT, pH 7.4, and stored in 50% EtOH at 4 °C.

Affinity Purification of Tetracysteine-Tagged Proteins Using Affinity Matrixes 6 and 7. The GFP with a *C*-terminal CCPGCC tag as well as a *N*-terminal his$_6$ tag was overexpressed

Scheme 1. Synthesis of Nonfluorescent SplAsH-Biotin 5[a]



[a] Reaction conditions: (a) H_2SO_4, MeOH; $BocHNCH_2CH_2NH_2HCl$, TEA, DMF; (b) $Hg(OAc)_2$, HOAc, EtOH/H_2O; (c) $AsCl_3$, DIPEA, $Pd(OAc)_2$, THF; ethandithiol, acetone/PBS; (d) TFA, DCM; (e) 6-((Biotinoyl)amino) hexanoic acid succinimidyl ester, TEA, DMF.

in *Escherichia coli* using standard methods.[5] The collected cell pellet was lysed in 250 mM KCl, 50 mM KPO_4, 1 mM EDTA, 10 mM 2-mercaptoethane-sulfonate (MES), and 1 mM tris-(carboxyethyl) phosphine (TCEP), pH 7.5, by sonication. The total protein concentration of the lysate was measured using EZQ protein quantitation kit (Life Technologies), and GFP concentration was quantified by GFP intrinsic fluorescence. The freshly prepared GFP lysate (1 mL, ~2.5 mg/mL) was added to 0.5 mL of affinity matrixes **6** and **7**, respectively. The resin was mixed at RT for 2 h by end-to-end rotation. The mixture was transferred to a short column, the unbound material was collected, and the resin was washed with buffer (250 mM KCl, 50 mM KPO_4, 1 mM EDTA, 10 mM MES, 1 mM TCEP, pH 7.5, 3 × 2 mL), and then eluted with buffer (250 mM KCl, 50 mM KPO_4, 1 mM EDTA, 10 mM 2,3-dimercapto-propanesulfonate (DMPS), 3 × 0.5 mL). Fractions were analyzed by SDS-PAGE (NuPAGE, 4−12% Bis-Tris Gel, Life Technologies).

Purification by the polyhistidine tag was done using the standard immobilized metal affinity chromatography method (Life Technologies), as follows. The freshly prepared GFP lysate (1 mL) in 50 mM NaH_2PO_4, 500 mM NaCl, pH 8.0, was incubated with 1 mL of Ni^{2+}-NTA resin at RT for 1 h by end-to-end rotation. The mixture was transferred to a short column, the unbound material was collected, and the resin was washed with buffer (50 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole, pH 8.0, 3 × 5 mL) and then eluted with buffer (50 mM NaH_2PO_4, 500 mM NaCl, 250 mM imidazole, pH 8.0, 3 × 0.5 mL). Fractions were analyzed by SDS-PAGE (NuPAGE, 4−12% Bis-Tris Gel, Life Technologies).

Kinetic Measurement of Binding Affinity of SplAsH-biotin 5 and Tetracysteine-Tagged Protein (GFP). The kinetic measurement of binding affinity of SplAsH-biotin **5** and tetracysteine-tagged protein (GFP) was carried out using the ForteBio system, which is a label-free, real-time detection system.[19] The streptavidin biosensor tips from ForteBio were prewet for 30 min in water prior to use. All interaction analyses were conducted at 25 °C in PBS buffer (pH 7.2) containing 5 mM MES, 0.1% BSA, and 0.05% Tween-20. The streptavidin biosensor tips were first incubated with SplAsH-biotin **5** (50 μM) to immobilize the SplAsH ligand onto the tips. Then, the SplAsH ligand coated tips were incubated with purified tetracysteine-tagged GFP (50 nM) in duplicate to generate the association curve. As a reference control, the SplAsH ligand coated tip was also incubated with a buffer solution. The dissociation curve was generated by incubating these tips with the PBS buffer described above. The *ForteBio Data Analysis Octet* software was used to process the curve by subtracting a sample curve from the reference curve, and the kinetic data were calculated by curve fitting.

RESULTS AND DISCUSSION

To improve the utility of carboxy-FlAsH-EDT_2 (CrAsH-EDT_2, Figure 1) in affinity purification of tetracysteine-tagged proteins,[11,20,21] our focus is to apply a photochemically stable FlAsH−EDT_2 analogue. Recently, a fluoro-substituted FlAsH (F_2FlAsH, Figure 1) has been reported to dramatically improve photostability.[22] However, the synthesis of F_2FlAsH gives poor overall yield (<4%), which limits its practical usefulness in applications in affinity purification.

We decided to focus on the use of a readily available nonfluorescent SplAsH **4** (Spirolactam Arsenical Hairpin binder, Scheme 1). SplAsH **4** was prepared according to the published procedure.[17] Briefly, fluorescein was converted to fluorescein methyl ester in MeOH using H_2SO_4 as a catalyst,[18] then the ester was reacted with mono Boc-protected ethylenediamine to give fluorescein spirolactam **1**.[23] The fluorescein spirolactam **1** was then converted to the bis-mercuric acetate derivative **2**[13] and then to the bis-arsenical derivative **3** by transmetalation with palladium acetate as a catalyst.[9] After cleavage of the Boc protecting group with TFA, SplAsH **4** was obtained in 63% overall yield in six steps. This synthesis can be easily scaled up for the preparation of multigram amounts of **4**. Compared to the synthesis of CrAsH-EDT_2 (16% overall yield),[11] the synthesis of SplAsH **4** has great advantages in terms of easy preparation and easy purification. The SplAsH **4** can be easily converted to SplAsH-biotin **5**. The absorbance maximum of SplAsH-biotin **5** was shifted from 509 to 242 nm with ε_{242} = 26 000 compared to FlAsH-EDT_2 (Figure 2), thus providing no inherent fluorescence whether or not it is bound to the tetracysteine motif.

To test the photochemical stability, CrAsH-EDT_2 and SplAsH-biotin **5** were each prepared in 100 μM concentration in degassed PBS buffer with 1 mM EDT, and 1 mL of each sample in a sealed quartz curvette was irradiated with a xenon arc lamp at 250 mW/cm^2. Samples were checked by HPLC every 30 min,

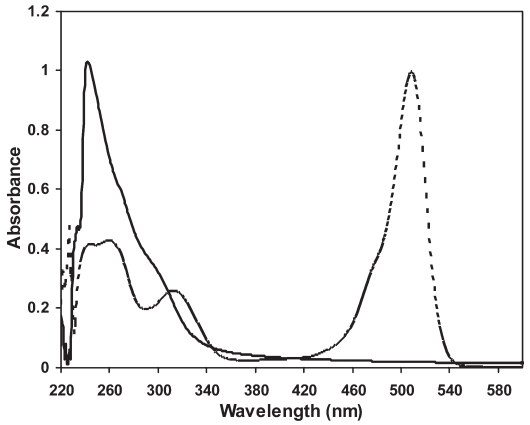


Figure 2. Absorbance spectra of FlAsH-EDT$_2$ (···) and SplAsH-biotin **5** (—) at PBS buffer.

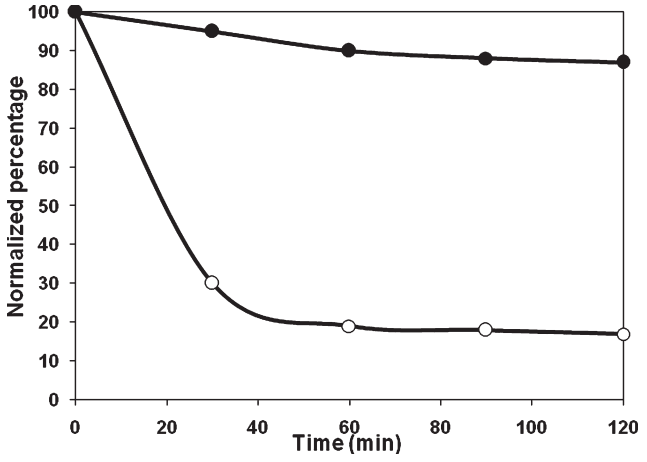


Figure 3. Photostability of CrAsH-EDT$_2$ (○) and SplAsH-biotin **5** (●) irradiated with a xenon arc lamp at 250 mW/cm^2.

and the results are shown in Figure 3. The results clearly show that the CrAsH-EDT$_2$ exhibits poor photochemical stability.[17] SplAsH-biotin **5** exhibits remarkably improved photochemical stability because blocking the quinoid tautomer formation by spirolactam formation eliminates the excited state that serves as an intermediate in the photobleaching process.[24]

In order to measure the binding affinity of SplAsH and tetracysteine-tagged protein, SplAsH-biotin **5** was first immobilized onto streptavidin sensor tips through biotin−streptavidin interaction. Then, the SplAsH coated sensor tips were incubated with tetracysteine-tagged GFP to generate the association curve, followed by incubating in buffer to get the dissociation curve (Figure 4). On the basis of the on-and-off rate, the dissociation constant K_D was calculated to be approximately 2.4 ± 1.2 nM. Compared to the dissociation constant K_D (~μM) for the interaction between His tag and Ni_2^+-NTA,[25] the SplAsH ligand has more than 1000-fold higher affinity (stronger, tighter binding) and thus should be much more useful for the capture and purification of low-abundance proteins.

To evaluate the application in affinity purification of tetracysteine-tagged proteins, the CrAsH-EDT$_2$ succinimidyl ester was coupled to Affigel-102 (Biorad), and the remaining free amines were capped with excess of *N*-acetoxysuccinimide.[11]

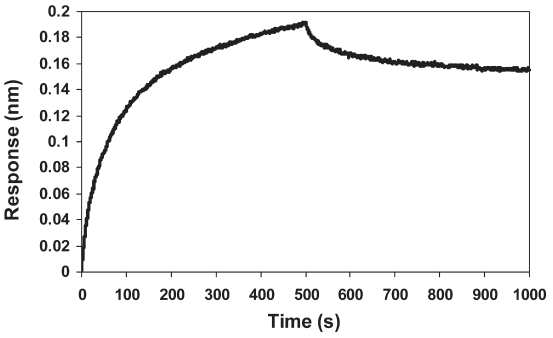


Figure 4. Kinetics of association and dissociation of SplAsH-biotin **5** and tetracysteine-tagged GFP.

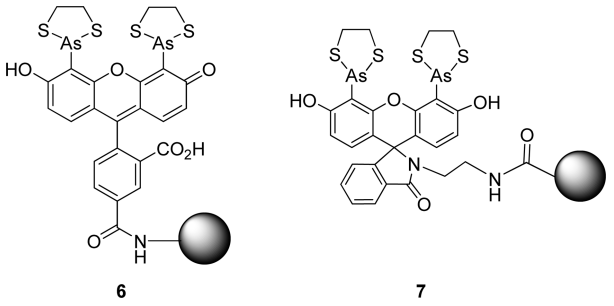


Figure 5. Affinity matrixes **6** and **7** used for purification of tetracysteine-tagged proteins.

SplAsH **4** was conjugated with Affigel-10 (Biorad), and unreacted NHS groups were quenched with ethanolamine. We chose the GFP with a *C*-terminal CCPGCC tag as well as a *N*-terminal his$_6$ tag as model test protein, because its recovery yield can be easily quantified by measuring the intrinsic fluorescence, and it can also be used to evaluate whether the two different purification methods will affect the GFP fluorescence property.[5,26] In separate, parallel experiments, the freshly prepared GFP lysate was mixed with CrAsH-EDT affinity matrix **6** and the new nonfluorescent affinity matrix **7** (Figure 5) in a buffer containing 250 mM KCl, 50 mM KPO_4, 1 mM EDTA, 10 mM MES, 1 mM TCEP, pH 7.5, for 2 h. The supernatant was collected from each, then each resin was washed with buffer, followed by eluting with buffer containing 10 mM DMPS. Fractions were analyzed by SDS-PAGE. For another comparsion, the same protein construct was purified by the conventional His$_6$:Ni^{2+}-NTA column.

It is well-known that the FlAsH ligand binds to the tetracysteine motif with high affinity and specificity, and the binding can be easily reversed by competing ligands, such as EDT, DTT, and DMPS.[11] These methods can be useful for the purification of native tetracysteine-tagged proteins. However, due to poor photochemical stability of CrAsH-EDT$_2$, the CrAsH-EDT affinity matrix **6** gave inconsistent results. Instead, SplAsH **4** displays superior photochemical stability. As shown in Figure 6, by using the new nonfluorescent affinity matrix **7** the desired protein was specifically captured and then eluted with DMPS to afford highly purified protein (90% purity) with reasonable yield (52% yield). Using the conventional His$_6$:Ni^{2+}-NTA method, the GFP was isolated in 92% purity and 70% recovery yield. The lower recovery yield is probably due to a stability issue of tetracysteine tag *in vitro*, by

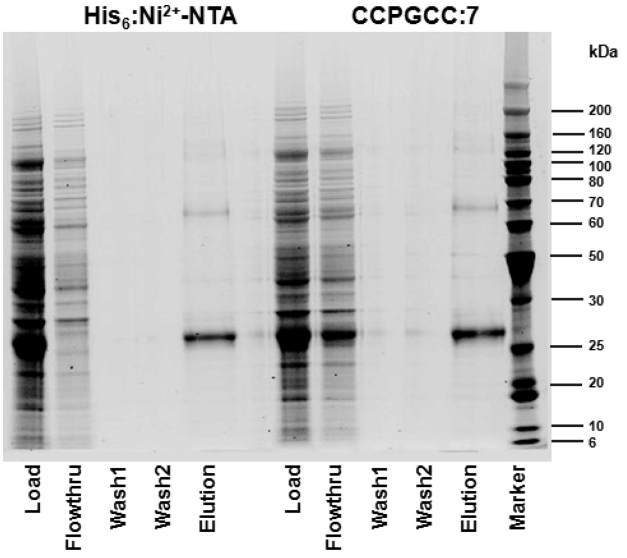


Figure 6. Affinity purification of a tetracysteine-tagged and his_6-tagged GFP using affinity matrix **7** and Ni^{2+}-NTA column. GFP lysate (load) was incubated with two supports, separated by pouring into columns (flowthrough), washed (wash), and eluted with either 10 mM DMPS or 250 mM imidazole (elution), respectively. The collected fractions were analyzed by a reducing 4−12% SDS-PAGE gel and stained with SYPRO-Ruby.

air oxidation and inactivation of the tetracysteine tag, and not due to instability of the new nonfluorescent affinity matrix **7**.

CONCLUSION

In summary, the superior photochemical stability of SplAsH **4** compared to CrAsH-EDT_2 can be applied for a new, improved method for protein affinity purification. SplAsH **4** can be easily prepared in large scale and high yield. SplAsH **4** retains high affinity and specificity for tetracysteine tags. This provides better affinity purification of tetracysteine-tagged proteins with SplAsH **4** than with CrAsH-EDT_2. In addition, SplAsH **4** is nonfluorescent whether or not it is bound to tetracysteine tags. This quenched property of SplAsH **4** provides an opportunity for potential surface-based applications by immobilizing SplAsH **4** onto beads or array surfaces to capture tetracysteine-tagged proteins, then performing protein detection, quantitation, and functional assays using dye-labeled detection antibody. Detailed studies of surface-based applications are currently underway.

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