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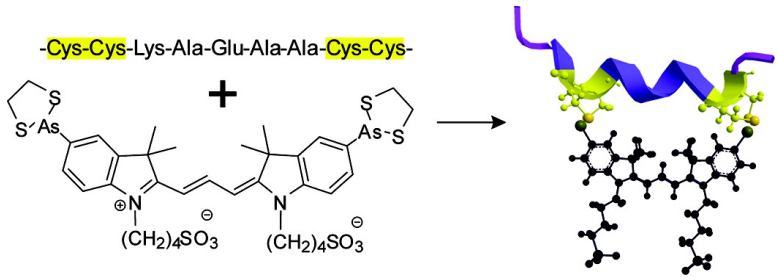

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A Red Cy3-Based Biarsenical Fluorescent Probe Targeted to a Complementary Binding Peptide

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Small-molecule biarsenical multiuse affinity probes (MAPs) FlAsH and ReAsH,[1,2] in conjunction with complementary protein tags, are important new tools for analyzing cellular function through live-cell imaging,[3,4] targeted protein inactivation,[5] and the measurement of protein dynamics and binding.[6] In addition, MAPs serve as affinity reagents for isolating intact protein complexes for complementary structural measurements.[7] These first-generation MAPs bind to a tetracoordinate arsenic group (TAG) binding motif (i.e., CCXXCC or FlAsHTAG) genetically engineered onto a protein of interest. They are superior to other targeted labeling strategies (such as the Halo-tag, the SNAP tag, and fluorescent proteins) in that the small peptide tag does not disrupt protein−protein interactions nor perturb the correct trafficking of tagged proteins.[8,9] The conserved interatomic distance (∼6 Å) between the two arsenic moieties in FlAsH and ReAsH complicates the selective labeling of multiple proteins with different reporters. To overcome these limitations, we have synthesized a new biarsenical MAP (i.e., AsCy3) based on Cy3, a member of the cyanine dye family, whose well-recognized brightness and photostability facilitate their utility in single-molecule measurements. The large interatomic distance between the two arsenics in AsCy3 (∼14.5 Å) coupled with the identification of a complementary high-affinity binding sequence CCKAEAACC (Cy3TAG) permits the simultaneous application of both AsCy3 and FlAsH to selectively label their respective binding TAGs in different proteins. In addition, the fluorescence of FlAsH overlaps with the absorption of AsCy3, which can act as an acceptor of fluorescence resonance energy transfer (FRET) to allow ratiometric measurements of protein association.

Synthesis of AsCy3 involved four steps (Scheme 1). Briefly, sulfonated Cy3[10] was prepared according to the procedure of Li et al.,[11] followed by mercuration and transmetalation to arsenic, which was liganded to ethanedithiol (EDT) for enhanced stability.[2,14] The overall yield of AsCy3 was 38%. Using 2D NMR to determine the structure of AsCy3 shows that the mercuration of the cyanine scaffold is specific with metal insertion *para* to nitrogen of the indoline ring system. Alternative synthetic routes, such as attaching the arsenic to the cyanine subunits prior to creating the linker region or coupling of the indolines under basic condition, resulted in substantially lower yields.

The inter-arsenic distance in AsCy3 (14.5 Å) was used in the design of a Cy3TAG binding sequence. On the basis of in silico distance measurements on NMR structures of α-helices[12] and hairpin-connected β-sheets, five different peptides were designed, and high-affinity binding was demonstrated for the Cy3TAG sequence CCKAEAACC (Figure 1, right panel). This sequence places two cysteine pairs two helical turns (i.e., ∼14 Å) apart, while the optimal FlAsH binding motif was based on one helical turn and is improved by the closer spacing provided by the proline−glycine turn.[2,13] Binding of AsCy3 is rapid and occurs in <15 s (see Supporting Information Figure S1). In comparison, FlAsH and

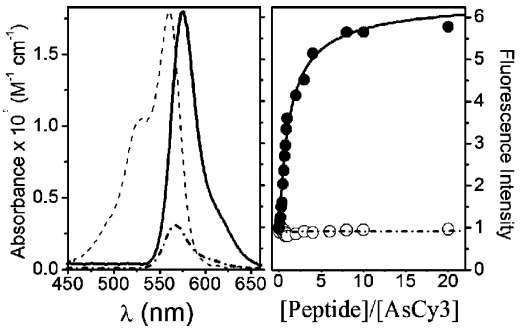
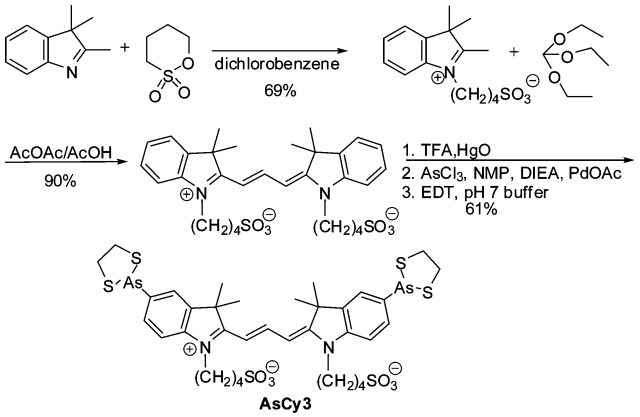


Figure 1. Left Panel: Identical absorbance spectra (- - -) and fluorescence emission spectra of AsCy3-EDT_2 (- · -) or the AsCy3-labeled Cy3TAG on the CaM-binding smMLCK peptide (—). Right Panel: Fluorescence enhancement of AsCy3 (1 μM) associated with addition of Cy3TAG on sm-MLCK (●) or control, deficient in Cy3TAG, sm-MLCK peptide (○).

Scheme 1. Synthesis of AsCy3



ReAsH binding under similar conditions occurs in a time scale of several minutes.[14]

The direct equimolar association between AsCy3 and the Cy3TAG can be fit assuming a simple Langmuir binding curve (Figure 1 and Supporting Information Figure S2). AsCy3 binds to the Cy3TAG with high affinity (apparent $K_d = 80 \pm 10$ nM) in the presence of 100 μM EDT, which is commonly used to reduce nonspecific binding, when labeling tagged proteins in cells.[13] The observed binding affinity of AsCy3 to the Cy3TAG is comparable to that for the association between FlAsH and FlAsHTAG in the presence of similar amounts of EDT,[27] whose reported dissociation constant in the absence of added EDT is ∼10 pM.[2,13]

AsCy3 has absorbance and fluorescence emission maxima at 560 and 568 nm, respectively. The absorbance spectrum is insensitive to AsCy3 binding to the Cy3TAG and the release of EDT, while the fluorescence spectrum is red-shifted ($\lambda_{max} = 576$ nm) with a concomitant 6-fold increase in the fluorescence quantum yield ($Q = 0.28$) (Figure 1, left panel). The peptide-bound AsCy3 conjugate has enhanced photostability in comparison to the corresponding

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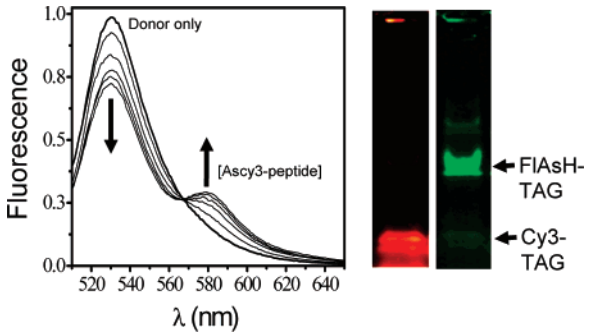

Figure 2. Left Panel: Fluorescence emission spectra of FlAsH-labeled CaM (1 μM) titrated with AsCy3-labeled smMLCK peptide from 0 to 1.0 μM in 0.2 μM increments. Right Panel: Labeling specificity of the FlAsHTAG in CaM and Cy3TAG in smMLCK reacted first with AsCy3, then FlAsH (all 1 μM). Same SDS-PAGE lane was imaged to detect AsCy3 (red) and FlAsH (green).

peptide conjugates involving FlAsH and ReAsH, retaining optimal spectral intensity for extended periods of time; in comparison to FlAsH and ReAsH, bleaching requires 3- and >30-fold more light intensity, respectively.[14] The fluorescence of AsCy3 is pH-independent between 4 and 9 (data not shown), permitting accurate ratiometric measurements in applications involving FRET between FlAsH and AsCy3 at neutral pH. Further, the large extinction coefficient (ϵ) of 180 000 M^{-1} cm^{-1} yields a brightness ($\epsilon \times Q = 5.0 \times 10^4$ M^{-1} cm^{-1}) that is similar to that of FlAsH ($4-6 \times 10^4$ M^{-1} cm^{-1})[2,13] and considerably higher than the red probe ReAsH ($1-3 \times 10^4$ M^{-1} cm^{-1}).[2,13] These properties suggest that AsCy3 will enjoy great utility in imaging applications.

The identification of a new small-molecule biarsenical probe with a TAG orthogonal to that of FlAsH has considerable significance for the simultaneous labeling of multiple tagged proteins for biophysical measurements of protein complex formation and live-cell imaging. The utility of AsCy3 was demonstrated with the same model system used previously to show the selectivity of FlAsH for live-cell imaging, that is, the calcium regulatory protein calmodulin (CaM) with an engineered FlAsHTAG binding sequence in helix A.[1] Binding specificity was assessed using a Cy3TAG on the CaM-binding sequence derived from smooth muscle myosin light chain kinase (i.e., smMLCK peptide), which forms a known high-affinity binding complex ($K_d = 1$ nM) with CaM.[15] Following the formation of the complex between CaM and smMLCK peptide (1 μM), AsCy3 (1 μM) and FlAsH (1 μM) were added sequentially prior to analysis by SDS-PAGE (Figure 2, right panel). The extent of labeling was imaged using appropriate filters, demonstrating considerable selectivity in the labeling of the FlAsHTAG in CaM by FlAsH (green) and the Cy3TAG on the smMLCK peptide by AsCy3 (red). A densitometric analysis of the labeling specificity indicates a >90% selectivity, indicating that FlAsH and AsCy3 can be used in the simultaneous labeling of different proteins for multicolor measurements. Further, binding can be directly measured by ratiometric methods suitable for cellular assays using AsCy3 as a FRET acceptor for FlAsH (Figure 2, left panel), where the Förster distance between this energy transfer pair is about 65 Å (see Supporting Information).

On the basis of our experience with the cellular imaging of biarsenicals[4] and the common usage of cyanine dyes to this purpose,[16] we believe that the new AsCy3 platform will perform as well as the first generation of biarsenicals for in vivo imaging and extend their utility to two-color measurements of protein complexes whose function is disrupted by large tags.

In conclusion, we have synthesized a new biarsenical fluorescent probe, AsCy3, that in conjunction with a high-affinity binding motif (i.e., Cy3TAG) (CCKAEAACC) permits specific labeling of the Cy3TAG in the presence of the previously identified FlAsHTAG (CCXXCC). Further, AsCy3 provides a FRET partner to the biarsenical dye FlAsH, permitting measurements of protein−protein interactions. AsCy3 has superior photostability and a minimal environmental sensitivity compared to the existing biarsenical probes FlAsH and ReAsH. Thus, the discovery of the new biarsenical probe AsCy3 provides an important next step in developing a whole toolkit of colored probes directed to different small binding motifs.

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Supporting Information Available: Experimental procedures for the synthesis of AsCy3, FRET and labeling experiments, expanded figures for binding and kinetics, and further references. This material is available free of charge via the Internet at http://pubs.acs.org.

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