Site-Specific Antibody Functionalization Using Tetrazine–Styrene Cycloaddition

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Supporting Information

ABSTRACT: Biologics, such as antibody–drug conjugates, are becoming mainstream therapeutics. Consequently, methods to functionalize biologics without disrupting their native properties are essential for identifying, characterizing, and translating candidate biologics from the bench to clinical practice. Here, we present a method for site-specific, carboxy-terminal modification of single-chain antibody fragments (scFvs). ScFvs displayed on the surface of yeast were isolated and functionalized by combining intein-mediated expressed protein ligation (EPL) with inverse electron-demand Diels–Alder (IEDDA) cycloaddition using a styrene–tetrazine pair. The high thiol concentration required to trigger EPL can hinder the subsequent chemoselective ligation reactions; therefore, the EPL reaction was used to append styrene to the scFv, limiting tetrazine exposure to damaging thiols. Subsequently, the styrene-functionalized scFv was reacted with tetrazine-conjugated compounds in an IEDDA cycloaddition to generate functionalized scFvs that retain their native binding activity. Rapid functionalization of yeast surface-derived scFv in a site-directed manner could find utility in many downstream laboratory and preclinical applications.

INTRODUCTION

Antibodies are a rapidly growing class of therapeutic agents with significant clinical success. In addition, imaging, diagnostics, and separation technologies often employ antibodies due to the high specificity and affinity for their cognate antigens. Functionalization of antibodies with chemical probes such as fluorophores, small-molecule drugs, or other biomolecules is used to customize these reagents for many applications. Still, a growing number of studies indicate that it is essential to append these probes in a site-specific manner that does not disrupt antibody function.

While other systems for site-specific labeling including FLASH, CLIP tags, and genetic code expansion (GCE) can also facilitate antibody functionalization, non-self-cleaving inteins are beneficial in that they exhibit traceless appendage of the functional group upon EPL. Although one can append a variety of functional groups to proteins using EPL, including post-translationally modified peptides, noncanonical amino acids, and biophysical probes, it can be advantageous to employ the expanding catalog of bioorthogonal reagents to append peptides, fluorophores, nanoparticles, and purification-enhancing moieties. To this end, we previously employed EPL with surface-displayed proteins to append an azide as a reactive handle for copper-catalyzed azide–alkyne cycloaddition (CuAAC). Although one can append certain CuAAC reactions: As an alternative, strained cyclooctynes (e.g., DBCO) could be used in copper free

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reactions with azides. A DBCO-cysteine reagent cannot be effectively used for the EPL-mediated scFv modification because strained cyclooctynes interact with thiols at concentrations that are orders of magnitude lower than those used in the EPL reaction. Instead, we have successfully used EPL to first append an azide that is subsequently reacted with a DBCO-conjugated probe to modify scFvs. However, certain applications such as in vivo targeting with an azide-modified scFv, followed by a DBCO probe would be limited since strained cyclooctynes suffer from poor bioavailability.

In order to address the aforementioned chemical compatibility issues with the yeast display EPL system, and to expand its utility, we explored the possibility of replacing Cu-catalyzed and strain-based biorthogonal reactions with an inverse electron-demand Diels–Alder (IEDDA) cycloaddition reaction. Styrene was shown to be inert to millimolar thiol concentration, enabling EPL driven carboxy-terminal modification of scFv with styrene. Subsequently, a styrene-modified scFv can be covalently linked to tetrazine-containing probes via IEDDA cycloaddition, and the functionalized scFvs retain their antigen-binding capacity.

RESULTS

Styrene is Compatible with Both EPL and IEDDA Reactions. The reaction of a tetrazine with trans-cyclooctyne (TCO) has become a well-established and useful tool in chemical biology as a result of its aqueous, rapid, and two-component nature. For compatibility with yeast surface display EPL, either tetrazine or TCO must be inert to high thiol concentrations (Figure 1, Steps A–C). To measure stability in the presence of free thiols, trans-cyclooctenol was incubated with FmocCysOH. After only 4 h, the trans-cyclooctenol had isomerized completely to the unreactive cis isomer (Figure 2a).

The dienophile in an IEDDA cycloaddition can also be activated by electron-donating groups instead of strain. One such activated alkene, 4-aminostyrene, possesses an amino group to both activate the alkene by donating electrons into the aryl system and act as a handle for derivatization. To test the compatibility of styrene with a high concentration of thiols, 4-aminostyrene was incubated with FmocCysOH and in contrast to trans-cyclooctenol, no degradation of the styrene was detectable after 12 h (Figure 2b). NMR spectroscopy was next used to examine the reaction kinetics of 4-acetamidostyrene (4) with a phenyltetrazine (Figure S1). The second-order rate constant was found to be $k = (4.0 \pm 0.1) \times 10^{-3}$ M$^{-1}$ s$^{-1}$.

Tetrazine is Not Compatible with a Yeast Display EPL Reaction. While a tetrazine appeared to be an appropriate dienophile for IEDDA in the context of a yeast display EPL reaction, the feasibility of using tetrazine in an EPL reaction was also explored. The tetrazine would have to withstand incubation with yeast and thiol for it to be compatible with the yeast display EPL reaction (one-pot reaction of Figure 1, Steps A–C) since it has been reported that a reduced tetrazine is no longer capable of an IEDDA reaction. Incubation of a tetrazine with live yeast cells resulted in a significant decrease in reactive tetrazine as measured by tetrazine absorbance (Figure 2c), indicating that the tetrazine is destroyed, sequestered, or reduced by the yeast cells. Tetrazine was also affected by common reducing agents used in intein-mediated EPL reactions (Figure 2d). Thiol-based reducing agents, including 2-mercaptoethanol, dithiothreitol, and MESNA, all drove tetrazine reduction. Incubation of a tetrazine with tris(2-carboxyethyl)phosphine (TCEP) at only 4 mM, the minimal amount required to release scFv from the yeast surface, also resulted in tetrazine reduction. Given that tetrazine reduction would hinder an IEDDA reaction with a styrene-probe reactant (Figure 1, Step D), its use in the EPL stages of the conjugation scheme was not pursued further.

Styrene Modification of scFv by EPL Followed by IEDDA Cycloaddition with a Tetrazine. Based on the results above, we chose to modify the scFv with the styrene reagent using EPL, and then derivatize the modified scFv with a tetrazine probe after removal of yeast and reducing agents (Figure 1). To test the strategy, an scFv that binds to fluorescein (4-4-20) was used. 4-4-20 scFv–intein fusion protein was displayed on the yeast surface, and the yeast was pelleted to remove growth medium. The yeast cells were resuspended, and 50 mM of MESNA was added, followed by 5 mM Cys–PEG$_3$–styrene (3) to promote the EPL reaction.
The EPL reaction proceeded for 3–18 h at room temperature with gentle shaking. MESNA released the Ag2p fusion and triggered intein cleavage, yielding an scFv with a carboxy-terminal thioester (Figure 1, Steps A and B). Subsequently, the Cys−PEG3−styrene reacted with the scFv-thioester to yield an scFv modified at its carboxy-terminus with styrene (Figure 1, Step C). After removal of yeast cells by pelleting, sterile-filtering through 0.22 μm spin columns, and dissolution in MESNA via buffer exchange with 10 000 Da MWCO filters, styrene-modified scFv was reacted with tetrazine−biotin in an IEDDA reaction to generate scFv functionalized with carboxy-terminal biotin (Figure 1, Step D). Western blotting demonstrated that biotin functionalization of scFv was dependent on both styrene modification and reaction with tetrazine−biotin (Figure 3a).

Figure 2. Stability of candidate reagents for EPL/IEDDA. (a) NMR spectra of vinyl protons of trans-cyclooctenol over the course of 4 h of incubation with FmocCysOH. (b) NMR spectra of 4-aminostyrene, FmocCysOH, and the reaction mixture after a 12 h incubation. (c) Absorbance at 525 nm of 5 mM tetrazine-amine with or without 100 μL of yeast cell culture incubated for 45 min. Mean and standard deviation are plotted from a minimum of three replicates. Inset: images showing the loss of the characteristic pink color of a tetrazine in the presence of yeast cells (*p < 0.01 using two tailed t-test). (d) Absorbance at 525 nm of a 5 mM tetrazine−amine solution containing various reducing agents used for yeast surface cleavage and EPL incubated for 45 min. Mean and standard deviation are plotted from a minimum of three replicates. Inset: images demonstrate loss of the characteristic pink color of a tetrazine (**p < 0.01 using ANOVA).

Figure 3. Functionalization of 4-4-20 scFv by EPL followed by IEDDA. (A) Western blot examining the biotin modification of a 4-4-20 scFv with an EPL reaction followed by reaction with 5 mM tetrazine−biotin overnight at 30 °C with gentle shaking. Total released and intein-cleaved scFv (α-FLAG) and functionalized scFv (α-Biotin) were probed. (B) Western blot showing EPL/IEDDA reaction yields as a function of MESNA concentration. α-FLAG detection is indicative of total antibody available for functionalization while α-biotin detection is used to compare the combined EPL and IEDDA efficiency. (C) Fraction of 4-4-20 scFv released from yeast that is biotinylated through combined EPL/IEDDA reactions. Total scFv released from yeast and the fraction of this scFv that was biotinylated as determined by binding to streptavidin beads were quantified by Western blotting with α-FLAG antibody. The mean and standard deviation for a minimum of three replicates is plotted. 78% ± 10% of the total scFv is modified using the combined EPL/IEDDA reaction (p < 0.05, t-test).
MESNA concentrations of 200 mM yielded the most robust combined EPL/IEDDA reaction as measured by the maximal amount of biotin-functionalized scFv (Figure 3b). The efficiency of the combined EPL/IEDDA reactions, in terms of the fraction of scFv released from the yeast surface that was successfully biotinylated, was 78% ± 10% (Figure 3c).

**ScFv Modification with Styrene−Tetrazine EPL/IEDDA Yields Functional scFvs.** Next, styrene-modified scFvs were conjugated to a fluorophore in an IEDDA reaction with tetrazine−Cy5 (Figure 1, Step D and inset). As demonstrated for the 4-4-20 scFv, antibody-associated Cy5 fluorescence required both styrene and tetrazine reactants (Figure 4a). To test that the scFv antigen-binding region retains function following modification, 4-4-20−Cy5 was applied to microtiter wells coated with FITC-dextran. There was a 50-fold increase in Cy5 signal for 4-4-20−Cy5 compared with an antibody that does not bind fluorescein, scFvA−49, indicating that the Cy5-modified 4-4-20 retained antigen-binding capability (Figure 4b). To confirm that antigen binding was not affected by EPL/IEDDA, the antigen binding affinities for unmodified 4-4-20 and 4-4-20−Cy5 scFvs were measured, and the $K_d$ values for 4-4-20−Cy5 (1.52 ± 0.05 nM) and...
unmodified 4-4-20 scFv (1.65 ± 0.04 nM) were indistinguishable (Figure 3c; p = 0.38, nonpaired, two tailed t-test).

As further verification of scFv functionalization and to demonstrate the utility of the EPL/IEDDA approach for cell-based assays, scFvA, a brain endothelial cell binding and internalizing antibody, was functionalized with Cy5. The scFvA–Cy5 conjugate was then used in flow cytometry and immunofluorescence based internalization assays. Rat brain endothelial cells (RBE4) were incubated with scFvA–Cy5 or control 4-4-20–Cy5, for 1 h at 37 °C to allow for scFv internalization. Internalization was quantified using flow cytometry, with 96% ± 3% of RBE4 cells having internalized scFvA–Cy5, whereas only ~1% of cells internalized 4-4-20 scFv–Cy5 (Figure 5a and b), indicating a specific interaction of scFvA–Cy5 with RBE4 cells. The internalization of scFvA was also evaluated using fluorescence microscopy. ScFvA–Cy5 was found localized in intracellular puncta (Figure 5c) as previously described. Staining patterns of scFvA–Cy5 and unmodified scFvA appear similar (Figure 5d), and a strong colocalization between antimyc and Cy5 signal was observed (Figure 5c) indicating internalization of intact scFvA–Cy5 conjugates.

**DISCUSSION**

We have described a facile method for functionalizing yeast-displayed scFv at their carboxy-terminus by combining EPL and IEDDA reactions. EPL modification presented challenges for the subsequent IEDDA reactions because of the high concentration of sulfur nucleophiles required for the EPL reaction. As a result, TCO was not an effective dienophile in our system due to its sensitivity to thiol reduction, but styrene was stable under the high thiol concentrations required of EPL.

Styrenes are known to undergo cycloaddition with tetrazines, and have been reported for the bioconjugation of DNA. However, taking advantage of the inertness of styrene to thiols in an EPL/IEDDA protein-labeling system has not yet been described previously. One shortcoming is that the rate of reaction between styrene and tetrazine (Figure S1, (4.0 ± 0.1) × 10−3 M−1 s−1) is quite slow compared to the reported reaction of TCO and tetrazine ((1−10) × 103 M−1 s−1). Using the system described here, the lowered reaction rate was overcome near to or within the antigen-binding domain. The utility of this method was also demonstrated using brain endothelial cell internalization assays with scFvA–Cy5, where the scFvA–Cy5 conjugate retains its ability to internalize into RBE4 cells. We believe that the facile, modular, site-directed antibody-labeling protocol demonstrated herein is a powerful means to facilitate the development and assessment of antibodies and biologics for laboratory and preclinical use.

**MATERIALS AND METHODS**

**Conditions.** All procedures were performed in air at ambient temperature (~22 °C) and pressure (1.0 atm) unless indicated otherwise.

**Chemical Synthesis.** Materials. Unless noted otherwise, reagents and solvents were from Sigma–Aldrich (Milwaukee, WI) and were used without further purification. Reagent-grade solvents: acetonitrile, dichloromethane (DCM), tetrahydrofuran (THF), and triethylamine (TEA) were dried over a column of alumina and were removed from a dry still under an inert atmosphere. Flash column chromatography was performed with 40−63 Å silica (230−400 mesh) from Silicycle (Québec City, Canada), and thin-layer chromatography was performed with EMD 250 μm silica gel 60 F254 plates.

**Solvent Removal.** The phrase “concentrated under reduced pressure” refers to the removal of solvents and other volatile materials using a rotary evaporator at water aspirator pressure (<20 Torr) while maintaining a water bath below 40 °C. Residual solvent was removed from samples at high vacuum (<0.1 Torr).

**Compound 1.** Boc-S-tert-butylthio-1-cysteine (500 mg, 1.6 mmol) from Chem-Impex International (Wood Dale, IL) was condensed under vacuum; 65 mg, 97%.
dissolved in THF (5 mL). N-Hydroxysuccinimide (186 mg, 1.6 mmol) and N,N′-dicyclohexylcarbodiimide (DCC; 363 mg, 1.7 mmol) were added, and the resulting solution was stirred overnight. The reaction mixture was filtered, and the filtrate was concentrated under reduced pressure. The residue was dissolved in DCM (15 mL). 4,7,10-Trioxa-1,13-tridecanedi-amine (0.9 mL, 4.3 mmol) was added, and the resulting solution was stirred overnight. The reaction mixture was filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by reverse-phase HPLC on a C18 column using a gradient of water–acetonitrile containing trifluoroacetic acid (0.1% v/v) to yield compound 1 as a clear oil (64 mg, 10% for 2 steps).

Compound 2. 4-Aminostyrene (100 mg, 0.8 mmol) was dissolved in DCM (8 mL). Succinic anhydride (84 mg, 0.84 mmol) and TEA (0.24 mL, 1.7 mmol) were added, and the resulting solution was stirred overnight. The reaction mixture was concentrated under reduced pressure. The residue was dissolved in EtOAc and washed twice with 1 M HCl. The organic layer was dried over Na₂SO₄(s) and concentrated under reduced pressure to yield compound 2 as a white solid (99 mg, 54%). 1H NMR (500 MHz, CD₃OD, δ): 7.52 (d, 2H, J = 8.6 Hz), 7.37 (d, 2H, J = 8.6 Hz), 6.65−6.71 (dd, 1H, J = 10.9 Hz, 17.7 Hz), 5.71 (d, 1H, J = 17.6 Hz), 5.15 (d, 1H, J = 11.0 Hz), 2.66 (s, 4H). 13C NMR (125 MHz, CD₃OD, δ): 176.4, 172.8, 139.6, 137.6, 137.5, 134.7, 127.6, 120.9, 32.3, 30.0. HRMS−ESI− (m/z): [M − H]− calcd for C₁₂H₁₃NO₃, 218.0823; found, 218.0821.

Cys(StBu)−PEG₃−styrene (3). Compound 1 (361 mg, 0.71 mmol) was dissolved in DCM (7 mL). Compound 2 (156 mg, 0.71 mmol), N-hydroxysuccinimide (82 mg, 0.71 mmol), and DCC (146 mg, 0.71 mmol) were added, and the resulting solution was stirred overnight. The reaction mixture was filtered, and then concentrated under reduced pressure. The residue was dissolved in acetonitrile and purified by reverse-phase HPLC on a C18 column using a gradient of water–acetonitrile containing trifluoroacetic acid (0.1% v/v) to yield compound 3 as a white solid (61 mg, 12% for 2 steps).

4-Acetamidostyrene (4). 4-Aminostyrene (100 mg, 0.84 mmol) was dissolved in DCM (8.4 mL). Acetyl chloride (0.18 mL, 0.84 mmol) and TEA (0.24 mL, 1.68 mmol) were added, and the resulting solution was stirred overnight. The reaction mixture was concentrated under reduced pressure, and the residue was dissolved in EtOAc. The solution was washed twice with 1 M HCl and twice with saturated aqueous NaHCO₃. The organic layer was dried over Na₂SO₄(s), and then concentrated under reduced pressure. The residue was purified further by chromatography on silica gel, eluting with 1:1 EtOAc/hexanes to yield compound 4 as a white solid (39 mg, 29%). 1H NMR (500 MHz, CDCl₃, δ): 7.47 (d, 2H, J = 8.6 Hz), 7.37 (d, 2H, J = 8.5 Hz), 7.14 (s, 1H), 6.70−6.64 (dd, 1H, J = 10.9, 17.6 Hz), 5.68 (d, 2H, J = 17.6 Hz), 5.20 (d, 2H, J = 10.9 Hz), 2.19 (s, 3H). 13C NMR (125 MHz, CDCl₃, δ): 168.1, 137.4, 136.1, 133.7, 126.8, 119.7, 113.1, 24.7. HRMS−ESI+ (m/z): [M + H]⁺ calcd for C₁₀H₁₁NO, 162.0913; found, 162.0912.

Styrene and trans-Cyclooctene Stability. Stock solutions were prepared by dissolving FmocCysOH, 4-acetamidostyrene (4), and trans-cyclooctenol in CD₃OD at a concentration of 200 mM. The solutions were combined in an NMR tube to give an equimolar ratio and mixed, and the tube was inserted immediately into an NMR spectrometer. A 16-scan 1H NMR spectrum was acquired every 60 min.
**Tetrazine—Styrene NMR Kinetics.** Stock solutions (6.25 mM in CD$_3$OD) were prepared of 5-[(4-1,2,4,5-tetrazin-3-yl)-benzylamino]-5-oxopentanoic acid and 4-acetamidostyrene (4). The solutions were combined in an NMR tube at an equimolar ratio and mixed, and the tube was inserted immediately into an NMR spectrometer. A 16-scan 1H NMR spectrum was acquired every 5 min. Conversion was monitored by disappearance of the tetrazine as determined by integration of the peak at 10.300–10.275 ppm. The integrity of the cycloaddition product (5) and its regioisomer (5') was assessed by LC–MS. The value of the second-order rate constant was determined by linear regression analysis of a plot of 1/[tetrazine] versus time.

**Yeast Surface Display.** Two distinct scFv’s were used in this work: 4-4-20 scFv (which binds to FITC and has an N-terminal FLAG tag) and scFvA (which binds to an antigen on the surface of RBE4 cells and has an N-terminal Myc tag). Fusions of these scFv’s to the 202–08 in the pCTrc vector, were transfected into EBY100 yeast cells as described previously.14 Transfected cells were grown at 30 °C in SD–CAA medium (20.0 g/L dextrose, 6.7 g/L yeast nitrogen base, 5.0 g/L casamino acids, 10.19 g/L Na$_2$HPO$_4$·7 H$_2$O, 8.56 g/L NaH$_2$PO$_4$·H$_2$O) as described previously.35,36,45 Cultures were induced with SD–CAA medium, that is identical to SD-CAA medium except dextrose is substituted for galactose at an induced with SGM. NaH$_2$PO$_4$ (0.7 M), casamino acids, 10.19 g/L Na$_2$HPO$_4$·7 H$_2$O, 8.56 g/L NaH$_2$PO$_4$·H$_2$O (as described previously). Yeast cultures were pelleted, and the cells were washed twice with 50 mM HEPES buffer, pH 7.2. For a typical reaction, 50 mL of yeast were pelleted and resuspended in 800 μL of 50 mM HEPES buffer, pH 7.2. One hundred microliters of 2 M MESNA solution was added to bring the final MESNA concentration to 200 mM. Immediately after MESNA addition, 100 μL of 50 mM Cys–PEG$_2$–styrene was added. These proportions can be scaled as needed to achieve the amount of modified scFv needed for downstream assays. The yeast reaction slurry was incubated for 1 h with gentle shaking at room temperature. Yeast cells were removed by centrifugation and secondary filtration through a 0.22 μm filter, to recover the styrene-modified scFv-containing supernatant. The reaction proceeds for an additional 2–17 h. After incubation, the supernatant was buffer exchanged four times with 50 mM HEPES buffer, pH 7.2, using 10 000 Da MWCO filters, to remove MESNA and excess styrene reactant (total exchange is 500 μL/wash). The scFv is concentrated using 10 000 Da MWCO filters to ≤50 μL. Next, 50 μL of 10× tetrazine probe (final concentration of 5 mM) is added to scFv-styrene (typically 45 μL scFv with 5 μL of tetrazine probe). The bioorthogonal conjugation step requires incubating the reaction overnight at 30 °C with gentle shaking. Finally, the reaction solution was then buffer-exchanged using 10 000 Da MWCO filters to remove excess tetrazine reactant. Two tetrazine probes were used in this work (Figure 1): tetrazine–biotin (Product number CP-6001 from Conju-Probe, San Diego, CA) and tetrazine–Cy5 (Product number 1189 from Click Chemistry Tools, Scottsdale, AZ).

**Incubation of Tetrazine with Live Yeast and Reducing Agents.** Live yeast were incubated with 5 mM of 3-(4-benzylamino)-1,2,4,5-tetrazine HCl (product number CP-6021-25MG from Conju-probe). After 45 min yeast was pellet and tetrazine absorbance was quantified at 525 nm using microplate reader. Similarly, 5 mM of phenyltetrazine functionized with an amine was incubated with MESNA (50 mM), TCEP (4 mM), BME (5%), or DTT (50 nM) for 45 min. After incubation, tetrazine absorbance at 525 nm was quantified using microplate reader.

**SDS–PAGE and Immunoblotting of Reacted Proteins.** scFv fractions were resolved using 4–12% w/v Bis-Tris acrylamide gels. Proteins were reduced and denatured by boiling samples in SDS-containing loading buffer with 5% v/v β-mercaptoethanol for 10 min. Proteins were transferred to nitrocellulose membranes. An anti-FLAG M2 monoclonal FLAG antibody (Sigma–Aldrich) and a BTN.1 antibody (Biologend) were used to probe membranes for scFv. Anti-mouse HRP secondary antibody (Jackson Laboratory, Bar Harbor, ME) was detected using ECL, and imaged using a Bio-Rad imaging system. A protein standard that is similar in size and that contained an N-terminal FLAG tag (Human IL17RB from Sino Biological, Beijing, China) was used to determine concentrations of scFv by immunoblotting.

**Quantifying Percentage of EPL/IDEAA Modified scFv.** 4-4-20 scFv was modified with biotin as described above. Next, scFv was incubated with magnetic streptavidin beads (Pierce) for 1 h at room temperature. For the total scFv samples the entire slurry was mixed with SDS loading buffer and boiled. Magnetic beads were removed and sample was resolved on 4–12% w/v bis-Tris acrylamide gel, transferred to nitrocellulose, and probed for scFv using anti-Flag antibody (Sigma). For the biotin-modified samples, magnetic beads were pulled down and washed 3 times to remove nonbound scFv. Beads were then resuspended, boiled, and quantified for scFv as described for the total scFv group. To account for background scFv binding of strep-beads to scFv, unmodified 4-4-20 was used and subtracted from each replicate to generate specific modification percentages. Western blot band intensity was quantified using ImageJ and normalized to total scFv signal.

**Solution-Phase Determination of scFv Modification with Cy5.** To determine modification of scFv by tetrazine-Cy5, 4-4-20 scFv was modified with styrene as described above in EPL/IDEAA section. Unreacted tetrazine-Cy5 was removed by buffer exchanging four times with 10 000 Da MWCO filters. Groups were then directly assessed for Cy5 fluorescence using a plate reader (Tecan).

**Solid-Phase Determination of scFv Modification with Cy5.** 4-4-20 and scFvA were modified with Cy5 using the approach described above. FITC-dextran was immobilized onto maxi-sorp ELISA plates (Nunc) by O.N. incubation at 4 °C. The next day, plates were washed two times with PBS + 0.05% w/v Tween 20 and blocked for 2 h with PBS + 1% w/v BSA. Plates were washed two times PBS + 0.05% w/v Tween 20 and 100 nM of scFv was added to each well and allowed to incubate at room temperature for 1 h. Plates were washed 5 times PBS + 0.05% w/v Tween 20 and Cy5 signal determined using a microtiter plate reader (Tecan).

**Fluorescein Quench Assay.** Modified and unmodified 4-4-20 scFv were titrated with fluorescein as described previously.14,40,44 Briefly, a 200 μL yeast display culture of 4-4-20 was split into two groups to generate 4-4-20 ± Cy5. The 4-4-20 concentration was determined using comparative Western blotting with known amounts of protein containing an N-terminal Flag tag. Samples were then incubated with fluorescein dilutions ranging from 0.08–40 nM. Fluorescein fluorescence was quantified using FluoroMax-3 fluorimeter (Horiba). Values of $K_d$ were determined as described previously.50
**Flow Cytometry.** RBE4 cells were cultured on collagen type I-coated tissue culture flasks in 45% v/v Alpha Minimum Essential Medium, 45% v/v Ham’s F10 medium, 10% v/v fetal calf serum, 100 mg/L streptomycin, 100 000 units/L penicillin G, 0.3 g/L Genetin, and 1 μg/L basic fibroblast growth factor (bFGF) as described previously.12 Cells were incubated with scFvA-Cys or unmodified scFvA (0.5 μM) for 1 h at 37 °C and 5% CO2. Cells were then washed three times with PBS (5 min washes) and trypsinized for 5 min at 37 °C and 5% CO2. Cultures were diluted 1:1 with serum-containing growth medium to quench trypsin, and the cells were pelleted by centrifugation. The cell pellet was resuspended in PBS containing 10 mM EDTA. Internalized fluorescence was measured with a BD FACS Caliber cytometer by quantifying 10 000 events/group using software from FlowJo (Ashland, OR).

**Fluorescence Microscopy.** RBE4 cells were cultured on glass coverslips.32 RBE4 cells were incubated with modified or unmodified scFvA (2 μM) for 1 h at 37 °C and 5% CO2. Cells were then washed three times with PBS, fixed in 4% v/v paraformaldehyde for 10 min, and permeabilized with 0.1% v/v Triton X-100. Permeabilized RBE4 cells were stained with 9E10 anti-Myc antibody (1:200), goat anti-mouse-AF488 conjugated antibody (1:200), and Hoechst 33342 (1:800). Slides were washed, mounted, and imaged on a Nikon upright fluorescence microscope.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.8b00114.

Disappearance of a tetrazine in the presence of 4-azidostyrene, 1H and 13C NMR spectra, as well as LC–MS chromatograms of synthetic compounds used in this study (PDF).

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Notes

The authors declare no competing financial interest.

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