Site-Specific Antibody Functionalization Using Tetrazine–Styrene Cycloaddition

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.

| Citation          | Umlauf, Benjamin J. et al. “Site-Specific Antibody Functionalization Using Tetrazine–Styrene Cycloaddition.” Bioconjugate chemistry 29 (2018): 1605-1613 © 2018 The Author(s) |
|-------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| As Published      | https://dx.doi.org/10.1021/ACS.BIOCONJCHEM.8B00114                                                                                                                                               |
| Publisher         | American Chemical Society (ACS)                                                                                                                                                                |
| Version           | Author’s final manuscript                                                                                                                                                                       |
| Citable link      | https://hdl.handle.net/1721.1/125600                                                                                                                                                            |
| Terms of Use      | Article is made available in accordance with the publisher’s policy and may be subject to US copyright law. Please refer to the publisher’s site for terms of use.                                      |
Site-Specific Antibody Functionalization Using Tetrazine–Styrene Cycloaddition

Benjamin J. Umlauf†, Kalie A. Mix‡,§, Vanessa A. Grosskopf†, Ronald T. Raines‡,#,§, and Eric V. Shusta†,*
†Department of Chemical and Biological Engineering, University of Wisconsin–Madison, Madison, Wisconsin 53706, United States
‡Department of Biochemistry, University of Wisconsin–Madison, Madison, Wisconsin 53706, United States
#Department of Chemistry, University of Wisconsin–Madison, Madison, Wisconsin 53706, United States
§Present Address: Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, United States.

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 pre-clinical 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 it is essential to append these probes in a site-specific manner that does not disrupt antibody function.

We previously developed a method for site-specific, carboxy-terminal antibody modification by employing yeast surface display in combination with expressed protein ligation (EPL). In this system, the carboxy-terminus of the scFv is fused to an engineered, non-self-cleavable intein (202-08) and expressed on the surface of yeast cells. Our laboratory previously generated 202-08 by evolving the Mxe GyrA intein to improve yeast surface display of scFv-intein fusions. Addition of a thiol, 2-mercaptoethanesulfonic acid (MESNA), liberates the scFv from the yeast surface and activates intein splicing to undergo transthioesterification and produce a carboxy-terminal thioester. Subsequently, a cysteine (Cys) amide is used to link the scFv to a Cys-modified probe of interest via an amide bond. Of note, this system enables rapid protein modification specifically at the carboxy-terminus of the released protein, without requiring protein purification. As a result, the yeast display EPL system is ideal for the rapid, high-throughput functionalization of antibodies.

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, non-canonical 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.\textsuperscript{45-48} 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).\textsuperscript{14} Although the rapid rate of this reaction makes it useful for many \textit{in vitro} applications, CuAAC can have more limited utility \textit{in vivo} due to the oxidative stress induced by Cu(I) and cross-reactivity of ascorbate with biological nucleophiles.\textsuperscript{20,21} Additionally, the multi-component nature of the reaction, requiring a copper catalyst, activating ligand, and reducing agent in addition to the azide and alkyne reagents often requires optimization to apply the reaction to different molecules and conditions. Further, the high concentration of thiols can impair certain CuAAC reactions.\textsuperscript{22-24,37} As an alternative, strained cyclooctynes (\textit{e.g.}, DBCO) could be used in copper free 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.\textsuperscript{25} Instead, we have successfully used EPL to first append an azide that is subsequently reacted with a DBCO-conjugated probe to modify scFvs.\textsuperscript{36} However, certain applications such as \textit{in vivo} targeting with an azide-modified scFv, followed by a DBCO probe would be limited since strained cyclooctynes suffer from poor bioavailability.\textsuperscript{52}

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 bioorthogonal 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.

\section*{RESULTS}

\subsection*{A Styrene is Compatible with Both EPL and IEDDA Reactions}

The reaction of a tetrazine with \textit{trans}-cyclooctene (TCO) has become a well-established and useful tool in chemical biology as a result of its aqueous, rapid, and two-component nature.\textsuperscript{26,27} 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, \textit{trans}-cyclooctenol was incubated with FmocCysOH. After only 4 h, the \textit{trans}-cyclooctenol had isomerized completely to the unreactive \textit{cis} isomer (Figure 2a). The dienophile in an IEDDA cycloaddition can also be activated by electron-donating groups instead of strain.\textsuperscript{28} One such activated alkene, 4-aminostyrene,\textsuperscript{29} 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 \textit{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.
The second-order rate constant was found to be \( k = (4.0 \pm 0.1) \times 10^{-3} \text{ M}^{-1}\text{s}^{-1} \).

### A Tetrazine is Not Compatible with a Yeast Display EPL Reaction

While a styrene 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 Aga2p fusion and triggered intein cleavage, yielding an scFv with a carboxy-terminal thioester (Figure 1, Steps A and B). Subsequently, the Cys–PEG\(_3\)–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 both on styrene modification and reaction with tetrazine–biotin (Figure 3a). 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 antigen in the form of 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<sup>49</sup>, 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; $p = 0.38$, non-paired, 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,<sup>49</sup> 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.<sup>49</sup> Staining patterns of scFvA-Cy5 and unmodified scFvA appear similar (Figure 5d), and a strong co-localization between anti-myc 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 scFvs 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.<sup>23</sup> 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,<sup>27,28,31,32</sup> and have been reported for the bioconjugation of DNA.<sup>31</sup> 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<sup>−3</sup> M<sup>−1</sup>s<sup>−1</sup>) is quite slow compared to the reported reaction of TCO and tetrazine (1–10 × 10<sup>3</sup> M<sup>−1</sup>s<sup>−1</sup>).<sup>27</sup> Using the system described here, the lowered reaction rate was overcome by adding an excess of tetrazine and performing the reaction at 30 °C.<sup>22</sup> Further tuning the electronics of each reagent could potentially enhance the reaction rates, and thereby enhance functionalization of low concentrations of styrene-modified proteins.<sup>29,33</sup> For instance, the rate could be accelerated...
by incorporation of electron-donating groups on the aryl ring of the styrene reagent, and by using dipyridyl-tetrazine in place of phenyltetrazine.

There exist several constraints with respect to the tetrazine moiety in the yeast display EPL/IEDDA system. Both live yeast cells and reducing agents, including the non-thiol based TCEP, resulted in reduction and subsequent inactivation of a tetrazine. To circumvent this problem, Cys–PEG$_3$–styrene (3) rather than Cys–PEG$_3$–tetrazine was used as the EPL reagent. It may be possible, however, to modify the scFv with Cys–PEG$_3$–tetrazine if reducing reagents were removed post-reaction and tetrazine re-oxidized. A recent study described incubation of tetrazines with methylene blue or using intense red light to re-oxidize a reduced tetrazine and restore reactivity. The both of these routes would, however, require additional steps; and thus the addition of styrene, rather than tetrazine, by EPL is preferable in this system. Given the relative differences in size between an scFv and styrene (~30 kDa versus ~100 Da), a PEG spacer between the cysteine amide used for EPL and the styrene moiety was added. In the experiments described here with biotin or fluorophore modification, the relatively small size of the tetrazine-probe is unlikely to cause major steric hindrance. Nevertheless, in applications that seek to modify the scFv–styrene with a tetrazine-probe of larger size (e.g., nanoparticulate substrates), the PEG spacer will likely have a beneficial impact.

Another important factor in method optimization was the concentration of MESNA employed in the EPL reaction. Interestingly, there exists an optimal EPL concentration of MESNA at 200 mM for maximum yields from the IEDDA reaction, despite the fact that additional scFv is released from the yeast surface when up to 800 mM MESNA is used. While one might expect a plateau rather than optimum in MESNA concentration, the presence of an optimum could be due to high concentrations of MESNA preventing the S- to N-acyl switch required for EPL modification with the styrene handle, or a result of competition between MESNA and cysteine for nucleophilic attack on the intein-generated thioester. At this optimum, we observed 78% ± 10% modification of 4-4-20 scFv using the EPL/IEDDA protocol, which is sufficient for downstream applications as demonstrated here. Finally, the utility of the yeast display EPL/IEDDA antibody modification method was demonstrated using a variety of fluorescence-based assays. Modification of 4-4-20 scFv with styrene enabled carboxy-terminal conjugation to tetrazine–Cy5 without affecting antigen binding. Such site-specific modification distal to the binding site can provide a distinct advantage over non-specific, amine- or thiol-based antibody functionalization protocols that often result in a large percentage of inactive antibody because probe functionalization occurs 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-L-cysteine (500 mg, 1.6 mmol) from Chem-Impex International (Wood Dale, IL) was 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-tridecanediamine (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 Na$_2$SO$_4$(s) and concentrated under reduced pressure to yield compound 2 as a white solid (99 mg, 54%). $^1$H NMR (500 MHz, CD$_3$OD, 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). $^{13}$C NMR (125 MHz, CD$_3$OD, 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 C$_{12}$H$_{13}$NO$_3$, 218.0823; found, 218.0821.

Cys(StBu)–PEG$_3$–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). The residue was then dissolved in 4.0 M HCl in dioxane, and the resulting solution was stirred for 1 h. The solution was sparged with N$_2$(g) for 10 min to remove HCl and then concentrated under reduced pressure 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 NaHCO$_3$. The organic layer was dried over Na$_2$SO$_4$, 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%). $^1$H NMR (500 MHz, CDCl$_3$, 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). $^{13}$C NMR (125 MHz, CDCl$_3$, 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 C$_{10}$H$_{11}$NO, 162.0913; found, 162.0912.

Styrene and trans-Cyclooctene Stability

Stock solutions were prepared by dissolving FmocCysOH, 4-acetamidostyrene (4), and trans-cyclooctenol in CD$_3$OD 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 $^1$H 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 $^1$H 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 $1/[\text{tetrazine}]$ 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 intein in the pCTre 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.$^{14,36,43}$ Cultures were induced with SG–CAA medium, that is identical to SD–CAA medium except dextrose is substituted for galactose at an OD$_{600nm}$ of 0.8–0.9. Cultures were induced for 48 h at 20 °C before harvesting the scFv from the yeast surface.

**EPL and IEDDA Cycloaddition of Yeast Surface-Displayed Proteins**

Yeast cultures were pelleted, and the cells were washed two times with 50 mM HEPES buffer, pH 7.2. For a typical reaction, 50 mL of yeast were pelleted and re-suspended 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$_3$–styrene is 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 filter to <50 μL. Next, 50 mM 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 minutes yeast was pelleted and tetrazine absorbance was quantified at 525 nm using microplate reader. Similarly, 5 mM of phenyltetrazine functionalized with an amine was incubated with MESNA (50 mM), TCEP (4 mM), BME (5%), or DTT (50 nM) for 45 minutes. 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 anti-biotin antibody (Biolegend) were used to probe membranes for scFv. Anti-mouse HRP secondary antibody (Jackson Laboratory, Bar Harbor, ME) was detected via 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 non-bound scFv. Beads were then re-suspended, 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 Image J 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 ELSIA 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. Briefly, a 200-mL 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.

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 geneticin, and 1 μg/L basic fibroblast growth factor (bFGF) as described previously. Cells were incubated with scFvA-Cy5 or unmodified scFvA (0.5 μM) for 1 h at 37 °C and 5% CO$_2$. Cells were washed three times with PBS (5-min washes) and trypsinized for 5 min at 37 °C and 5% CO$_2$. 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 FACSCaliber cytometer by quantifying 10,000 events/group using software from FlowJo (Ashland, OR).

Fluorescence Microscopy

RBE4 cells were cultured on glass coverslips. RBE4 cells were incubated with modified or unmodified scFvA (2 μM) for 1 h at 37 °C and 5% CO$_2$. 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 antibody (1:200), and Hoechst 33342 (1:800). Slides were washed, mounted, and imaged on a Nikon upright fluorescence microscope.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.
Acknowledgments

K.A.M. was supported by Molecular Biosciences Training Grant T32 GM007215 (NIH) and a fellowship from the University of Wisconsin–Madison College of Agricultural and Life Sciences. This work was funded by National Science Foundation grant CBET1403350.

References

1. Pleiner T, Bates M, Trakhanov S, Lee CT, Schliep JE, Chug H, Böhning M, Stark H, Urlaub H, Görlich D. Nanobodies: Site-specific labeling for super-resolution imaging, rapid epitope-mapping and native protein complex isolation. eLife. 2015; 4:e11349. [PubMed: 26633879]

2. Devaraj NK, Upadhyay R, Huan JB, Hilderbrand SA, Weissleder R. Fast and sensitive pretargeted labeling of cancer cells through a tetrazine/trans-cyclooctene cycloaddition. Angew Chem Int Ed. 2009; 48:7013–7016.

3. Casi G, Huguenin-Dezot N, Zuberbühler K, Scheuermann J, Neri D. Site-specific traceless coupling of potent cytotoxic drugs to recombinant antibodies for pharmacodelivery. J Am Chem Soc. 2012; 134:5887–5892. [PubMed: 22994212]

4. Parslow AC, Parakh S, Lee FT, Gan HK, Scott AM. Antibody–drug conjugates for cancer therapy. Biomedicines. 2016; 4:14–20.

5. Sievers EL, Senter PD. Antibody–drug conjugates in cancer therapy. Annu Rev Med. 2013; 64:15–29. [PubMed: 23043493]

6. Bernardes GJL, Steiner M, Hartmann I, Neri D, Casi G. Site-specific chemical modification of antibody fragments using traceless cleavable linkers. Nat Protoc. 2013; 8:2079–2089. [PubMed: 24091555]

7. Xiao H, Woods EC, Vukojicic P, Bertozzi CR. Precision glycocalyx editing as a strategy for cancer immunotherapy. Proc Natl Acad Sci USA. 2016; 113:10304–10309. [PubMed: 27551071]

8. Schaefer G, Haber L, Crocker LM, Shia S, Shao L, Dowbenko D, Totpal K, Wong A, Lee CV, Stawicki S, Clark R, et al. A two-in-one antibody against HER3 and EGFR has superior inhibitory activity compared with monospecific antibodies. Cancer Cell. 2011; 20:472–486. [PubMed: 22014573]

9. Junutula JR, Raab H, Clark S, Bhakta S, Leipoldt DD, Weir S, Chen Y, Simpson M, Tsai SP, Dennis MS, et al. Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. Nat Biotechnol. 2008; 26:925–932. [PubMed: 18641636]

10. Strop P, Delaria K, Foletti D, Witt JM, Hasa-Moreno A, Poulsen K, Casas MG, Dorywalska M, Farias S, Pios A, et al. Site-specific conjugation improves therapeutic index of antibody drug conjugates with high drug loading. Nat Biotechnol. 2015; 33:694–696. [PubMed: 26154005]

11. Mårlind J, Kaspar M, Trachsel E, Sommavilla R, Hindle S, Bacci C, Giovannoni L, Neri D. Antibody-mediated delivery of interleukin-2 to the stroma of breast cancer strongly enhances the potency of chemotherapy. Clin Cancer Res. 2008; 14:6515–6524. [PubMed: 18927291]

12. Gutbrodt KL, Schliemann C, Giovannoni L, Frey K, Pabst T, Klapper W, Berdel WE, Neri D. Antibody-based delivery of interleukin-2 to neovasculature has potent activity against acute myeloid leukemia. Sci Transl Med. 2013; 5:201ra118.

13. Muir TW, Sondhi D, Cole PA. Expressed protein ligation: A general method for protein engineering. Proc Natl Acad Sci USA. 1998; 95:6705–6710. [PubMed: 9618476]

14. Marshall CJ, Agarwal N, Kalia J, Grosskopf VA, McGrath NA, Abbott NL, Raines RT, Shusta EV. Facile chemical functionalization of proteins through interein-linked yeast display. Bioconjug Chem. 2013; 24:1634–1644. [PubMed: 23924245]

15. Shusta EV, Holler PD, Kieke MC, Kranz DM, Wittrup KD. Directed evolution of a stable scaffold for T-cell receptor engineering. Nat Biotechnol. 2000; 18:754–759. [PubMed: 10888844]

16. Huang D, Gore PR, Shusta EV. Increasing yeast secretion of heterologous proteins by regulating expression rates and post-secretory loss. Biotechnol Bioeng. 2008; 101:1264–1275. [PubMed: 18781684]

17. Tillotson BJ, Lajoie JM, Shusta EV. Yeast display-based antibody affinity maturation using detergent-solubilized cell lysates. Methods Mol Biol. 2015; 1319:65–78. [PubMed: 26060070]
18. Valiyaveetil FI, Sekedat M, MacKinnon R, Muir TW. Glycine as a D-amino acid surrogate in the K⁺-selectivity filter. Proc Natl Acad Sci USA. 2004; 101:17045–17049. [PubMed: 15563591]

19. Flavell RR, Kothari P, Bar-Dagan M, Synan M, Vallabhajosula S, Friedman JM, Muir TW, Ceccarini G. Site-specific ¹⁸F-labeling of the protein hormone leptin using a general two-step ligation procedure. J Am Chem Soc. 2008; 130:9106–9112. [PubMed: 18570424]

20. Brewer GJ. Risks of copper and iron toxicity during aging in humans. Chem Res Toxicol. 2010; 23:319–326. [PubMed: 19968254]

21. Reihl O, Lederer MO, Schwack W. Characterization and detection of lysine–arginine cross-links derived from dehydroascorbic acid. Carbohydr Res. 2004; 339:483–491. [PubMed: 15013385]

22. Hong V, Presolski SI, Ma C, Finn MG. Analysis and optimization of copper-catalyzed azide–alkyne cycloaddition for bioconjugation. Angew Chem Int Ed. 2009; 48:9879–9883.

23. McKay, Craig S., Finn, MG. Click chemistry in complex mixtures: Bioorthogonal bioconjugation. Chem Biol. 2014; 21:1075–1101. [PubMed: 25237856]

24. Presolski SI, Hong VP, Finn MG. Copper-catalyzed azide–alkyne click chemistry for bioconjugation. Curr Protoc Chem Biol. 2009; 3:153–162.

25. Beatty KE, Fisk JD, Smart BP, Lu YY, Szychowski J, Hangauer MJ, Baskin JM, Bertozzi CR, Türell DA. Live-cell imaging of cellular proteins by a strain-promoted azide–alkyne cycloaddition. ChemBioChem. 2010; 11:2092–2095. [PubMed: 20836119]

26. Taylor MT, Blackman ML, Dmitrenko O, Fox JM. Design and synthesis of highly reactive dienophiles for the tetracene–trans-cyclooctene ligation. J Am Chem Soc. 2011; 133:9646–9649. [PubMed: 21599005]

27. Blackman ML, Royzen M, Fox JM. Tetracene ligation: Fast bioconjugation based on inverse-electron-demand Diels–Alder reactivity. J Am Chem Soc. 2008; 130:13518–13519. [PubMed: 18798613]

28. Wijnen JW, Zavarise S, Engberts JBFN. Substituent effects on an inverse electron demand hetero Diels–Alder reaction in aqueous solution and organic solvents: Cycloaddition of substituted styrenes to di(2-pyridyl)-1,2,4,5-tetrazine. J Org Chem. 1996; 61:2001–2005.

29. Knall AC, Hollauf M,Slugovc C. Kinetic studies of inverse electron demand Diels–Alder reactions (iEDDA) of norbornenes and 3,6-dipyrindin-2-yl-1,2,4,5-tetrazine. Tetrahedron Lett. 2014; 55:4763–4766. [PubMed: 25125544]

30. Zhang H, Trout WS, Liu S, Andrade GA, Hudson DA, Scinto SL, Dicker KT, Li Y, Lazouski N, Rosenthal J, et al. Rapid bioorthogonal chemistry turn-on through enzymatic or long wavelength photocatalytic activation of tetracene ligation. J Am Chem Soc. 2016; 138:5978–5983. [PubMed: 27078610]

31. Rieder U, Luedtke NW. Alkene–tetracene ligation for imaging cellular DNA. Angew Chem Int Ed. 2014; 126:9322–9326.

32. Sauer J, Heldmann DK, Hetzenegger J, Krauthan J, Schicht H, Schuster J. 1,2,4,5-Tetrazine: Synthesis and reactivity in [4+2] cycloadditions. Eur J Org Chem. 1998:2885–2896.

33. Knall AC, Slugovc C. Inverse electron demand Diels–Alder reactions (iEDDA)-initiated conjugation: A potential click chemistry scheme. Chem Soc Rev. 2013; 42:5131–5142. [PubMed: 23563107]

34. Brown KC. Peptidic tumor targeting agents: The road from phage display peptide selections to clinical applications. Curr Pharm Des. 2010; 16:1040–1054. [PubMed: 20030617]

35. Li S, Gray BP, McGuire MJ, Brown KC. Synthesis and biological evaluation of a peptide–paclitaxel conjugate which targets the integrin αvβ6. Bioorg Med Chem. 2011; 19:5480–5489. [PubMed: 21868241]

36. Marshall CJ, Grosskopf VA, Moehling TJ, Tillotson BJ, Wiepz GJ, Abbott NL, Raines RT, Shusta EV. An evolved Mxe GyrA intein for enhanced production of fusion proteins. ACS Chem Biol. 2015; 10:527–538. [PubMed: 25384269]

37. Lin PC, Ueng SH, Tseng MC, Ko JL, Huang KT, Yu S-C, Adak AK, Chen YJ, Lin C-C. Site-specific protein modification through Cu(I)-catalyzed 1,2,3-triazole formation and its implementation in protein microarray fabrication. Angew Chem Int Ed. 2006; 118:4392–4396.
38. Sydor JR, Mariano M, Sideris S, Nock S. Establishment of intein-mediated protein ligation under denaturing conditions: C-Terminal labeling of a single-chain antibody for biochip screening. Bioconjugate Chem. 2002; 13:707–712.

39. Albertsen L, Shaw AC, Norrild JC, Strømgaard K. Recombinant production of peptide C-terminal α-amides using an engineered intein. Bioconjugate Chem. 2013; 24:1883–1894.

40. Boder ET, Midelfort KS, Wittrup KD. Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. Proc Natl Acad Sci USA. 2000; 97:10701–10705. [PubMed: 10984501]

41. Wang XX, Shusta EV. The use of scFv-displaying yeast in mammalian cell surface selections. J Immunol Methods. 2005; 304:30–42. [PubMed: 16099466]

42. Shindo A, Maki T, Mandeville ET, Liang AC, Egawa N, Itoh K, Itoh N, Borlongan M, Holder JC, Chuang TT, McNeish JD, Tomimoto H, Lok J, Lo EH, Arai K. Astrocyte-derived pentraxin 3 supports blood–brain barrier integrity under acute phase of stroke. Stroke. 2016; 47:1094–1100. [PubMed: 26965847]

43. Zhang X, Wang XX, Shusta EV. Creation and evaluation of a single-chain antibody tetramer that targets brain endothelial cells. AIChE J. 2014; 60:1245–1252. [PubMed: 24659822]

44. Tillotson BJ, Cho YK, Shusta EV. Cells and cell lysates: A direct approach for engineering antibodies against membrane proteins using yeast surface display. Methods. 2013; 60:27–37. [PubMed: 22449570]

45. Elias DR, Cheng Z, Tsourkas A. An intein-mediated site-specific click conjugation strategy for improved tumor targeting of nanoparticle systems. Small. 2010; 6:2460–2468. [PubMed: 20925038]

46. Sen Gupta S, Kuzelka J, Singh P, Lewis WG, Manchester M, Finn MG. Accelerated bioorthogonal conjugation: A practical method for the ligation of diverse functional molecules to a polylvalent virus scaffold. Bioconjugate Chem. 2005; 16:1572–1579.

47. Bundy BC, Swartz JR. Site-specific incorporation of p-propargyloxyphenylalanine in a cell-free environment for direct protein–protein click conjugation. Bioconjugate Chem. 2010; 21:255–263.

48. Li X, Patterson JT, Sarkar M, Pedzisa L, Kodadek T, Roush WR, Rader C. Site-specific dual antibody conjugation via engineered cysteine and selenocysteine residues. Bioconjugate Chem. 2015; 26:2243–2248.

49. Wang XX, Cho YK, Shusta EV. Mining a yeast library for brain endothelial cell-binding antibodies. Nat Methods. 2007; 4:143–145. [PubMed: 17206151]

50. Burns ML, Malott TM, Metcalf KJ, Hackel BJ, Chan JR, Shusta EV. Directed evolution of brain-derived neurotrophic factor for improved folding and expression in Saccharomyces cerevisiae. Appl Environ Microbiol. 2014; 80:5732–5742. [PubMed: 25015885]

51. Carter PJ, Lazar GA. Next generation antibody drugs: Pursuit of the ‘high-hanging fruit’. Nat Rev Drug Discov. 2018; 17:197–223. [PubMed: 29192287]

52. Chang PV, Prescher JA, Sletten EM, Baskin JM, Miller IA, Agard NJ, Lo A, Bertozzi CR. Copper-free click chemistry in living animals. Proc Natl Acad Sci U S A. 2010; 107:1821–1826. [PubMed: 20080615]
Figure 1.
Route for the site-specific functionalization of a yeast surface-displayed scFv. A four-step process is used to functionalize the carboxy-terminus of an scFv by coupling expressed protein ligation (EPL) and inverse electron-demand Diels–Alder (IEDDA) cycloaddition. Step A is the release of the scFv from the yeast surface by thiol–disulfide exchange with MESNA. Step B is the cleavage of the intein and formation of a thioester with MESNA at the carboxy-terminus. Step C is reaction of the thioester with Cys-PEG$_3$-styrene (3). Steps A–C occur in a one-pot reaction with yeast, MESNA and Cys-PEG$_3$-styrene (3). After removal of yeast and MESNA, Step D is the IEDDA conjugation between scFv–styrene and a tetrazine-containing probe. Inset: structures of two tetrazine probes used for IEDDA cycloaddition.
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).
Figure 4. Evaluation of 4-4-20 scFv function after EPL/IEDDA with styrene-tetrazine

(a) 4-4-20 scFv-associated Cy5 signal after EPL/IEDDA modification with Cys–PEG₃–
styrene (3) and tetrazine–Cy5. Unreacted tetrazine-Cy5 is removed from the scFv fraction using buffer exchange and the remaining Cy5 fluorescence measured (*p < 0.01 using ANOVA). The mean and standard deviation for a minimum of three replicates for each group is plotted. (B) 4-4-20 and scFvA modified with Cy5 are incubated with immobilized FITC–dextran antigen. Unbound scFv was removed and wells quantified for Cy5 signal (*p < 0.01, t-test). The mean and standard deviation for a minimum of three replicates is plotted. (C) An equilibrium binding analysis by fluorescence quench affinity titration was used to compare the binding affinity of 20 nM unmodified 4-4-20 or Cy5-modified 4-4-20. Calculated Kₐ values (mean ± SD) are 1.65 ± 0.04 nM and 1.52 ± 0.05 nM, respectively (p = 0.38 using non-paired, two-tailed t-test).
Figure 5. Internalization of Cy5-labeled scFv into rat brain endothelial (RBE4) cells
(a) Representative flow cytometry dot plot after RBE4 cell internalization of scFvA-Cy5 (blue) or 4-4-20-Cy5 (red), with sample gate used for quantification. (b) Quantification of Cy5-positive cells across three independent experiments for each group, with mean and standard deviation plotted (*p < 0.01, t-test). (c) Fluorescence microscopy images of RBE4 cells after allowing for internalization of scFvA–Cy5 (red). Cells were fixed, permeabilized and stained for a Myc tag on the scFv (green). Nuclei were stained with Hoechst 33342 (blue) (d) RBE4 cells treated with scFvA–styrene that was not reacted with tetrazine–Cy5 and is stained as in panel F. (e) RBE4 cells not treated with any scFv, and stained as in panel c. Scale bars = 20 μm.