Creation and Evaluation of a Single-Chain Antibody Tetramer that Targets Brain Endothelial Cells

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Antibodies that target and internalize into blood-brain barrier (BBB) endothelial cells offer promise as drug delivery agents. Previously, we identified a single-chain antibody (scFvA) capable of binding to the BBB. In an attempt to improve the binding and internalization properties of scFvA, a biotinylation tag (Avitag) was fused to scFvA and the protein secreted by yeast. The scFvA-Avitag could be biotinylated by yeast-displayed BirA enzyme and biotinylated scFvA-Avitag could be used to create scFv tetramers. Tetramerization of scFvA improved the internalization of scFvA into BBB endothelial cells, and biotinylated scFvA-Avitag could also be used to target streptavidin-coated quantum dots for BBB endothelial cell internalization. Perfusing the rat brain with scFvA-tetramer confirmed that the antigen targeted by scFvA is distributed on the blood side of the BBB, suggesting the potential for downstream application of scFvA in brain-targeted drug delivery. © 2014 American Institute of Chemical Engineers *AIChE J*, 60: 1245–1252, 2014
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Introduction

Treatment of central nervous system disease is a substantial challenge owing to the presence of the blood-brain barrier (BBB). This endothelial barrier restricts the diffusion of small molecules into the brain and forces most molecules to cross the BBB by specific carrier- or receptor-mediated transport systems.[1,2] To facilitate drug delivery into brain for neurological disease therapy, various studies have been performed to identify BBB-resident receptor-mediated transport systems and cognate targeting antibodies that can be used for brain-targeted drug delivery.[3] For such a so-called transcytosis system to work, the targeting antibody needs to bind the brain endothelial cell surface on the blood side of the BBB, internalize into the vesicular transport pathway, traffic through the cytoplasm and ultimately release on the brain side.[3] Given the key role for internalization, we recently identified an antibody that could target an endocytosing BBB receptor in a rat brain endothelial cell line (RBE4).[4] As a function of the screening platform, the antibody was in the form of a single-chain antibody fragment (scFv) and as such was not optimal in terms of affinity (~80 nM) or its capability to cluster targeted receptors for the efficient initiation of endocytosis.[4] As multimerization, particularly tetramerization, can increase the binding avidity for a cell surface,[5] and binding of multiple cell surface receptors can help activate the internalization process,[6–8] it was desired to further explore the synthesis of scFv tetramers.

To this end, several approaches have been used to prepare protein and peptide tetramers, such as adjusting the linker length between the heavy and light chains of scFvs,[9,10] secreting the antibody in a designed tetramer format[11] or expressing as an scFv-streptavidin fusion that will spontaneously form tetramers via streptavidin (SA) interactions.[12–14] As an alternative, one can take advantage of the tetrameric nature of avidin or SA along with its strong affinity for biotin, first biotinylating the target protein and then combining with SA to form tetramers.[15] This approach has been well studied and possesses several advantages. The high affinity interaction between biotin and SA ($Kd = 5 \times 10^{-15}$ M) renders resultant tetramers quite stable. Moreover, the target protein can be biotinylated by appending a short peptide sequence known as an Avitag[16,17] to the target protein and reacting with the BirA biotinylation enzyme. As the Avitag leads to site-specific biotinylation, it tends to be less deleterious to protein function compared with, for example, *N*-Hydroxysuccinimide ester chemistry which leads to nonspecific functionalization of primary amines throughout the protein.[18] Finally, the monobiotinylated protein can be easily conjugated to SA or modified SA forms such as fluorophore-conjugated SA for imaging purposes[19,20] or therapeutic-conjugated SA for targeted drug delivery.[21,22]

In this study, the aforementioned BBB targeting antibody, scFvA,[4] was modified by introduction of an Avitag, and the fusion protein secreted from yeast. Purified Avitag-scFvA was subsequently biotinylated using yeast surface displayed BirA.[23] Biotinylated antibody was combined with SA to form scFvA-tetramers that can bind and internalize efficiently into brain endothelial cells *in vitro* and bind to the brain microvasculature *in vivo* upon brain perfusion.

Materials and Methods

Reagents and buffers

All chemical reagents were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma-Aldrich (St. Louis, MO)

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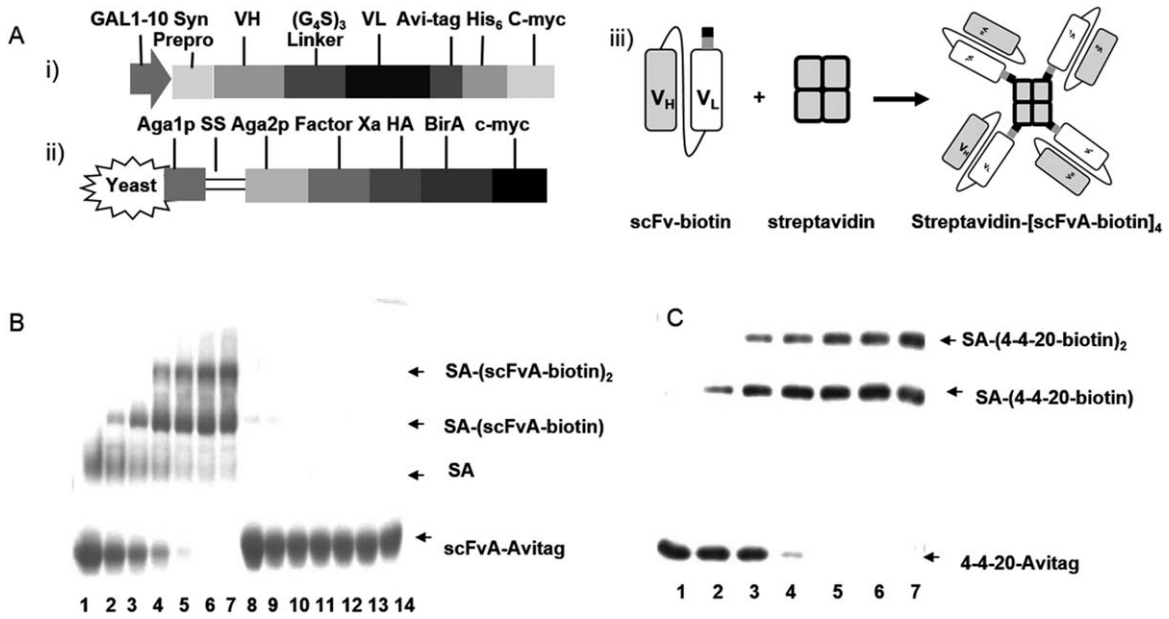

Figure 1. Secretion and biotinylation of scFvA-Avitag proteins.

(A) Construct schematics (i) scFv-Avitag expression cassette, (ii) BirA expression on yeast surface along with HA and c-*myc* epitope tags, and (iii) tetramer preparation. Depending on the ratio of biotinylated scFv-Avitag to SA, monomers, dimers, and trimers can also be formed. (B) The scFvA-Avitag biotinylation product was removed at different times points, mixed with SA, and the resultant product resolved by SDS-PAGE. The gel was stained with Coomassie blue. Lanes 1–7: biotinylation product at 0.5, 1, 2, 3, 4, 6, 8 h. Lanes 8–14, sample sequence the same as lanes 1–7. Samples 1–7 were mixed with SDS-containing sample buffer without DTT and without boiling. Under these conditions, the tetrameric state of SA and the interaction of SA with biotin are preserved so one can observe scFvA-Avitag, SA, and SA-scFvA-Avitag multimers as indicated. In contrast, samples 8–14 were mixed with SDS-containing sample buffer with DTT and boiling 3 min. Under these conditions, the scFv-SA multimers are not stable, nor is the tetrameric state of SA. Thus, in Lanes 8–14, only the purified scFvA-Avitag is visualized. (C) Temporal evaluation of 4-4-20-Avitag biotinylation status. Lanes 1–7, same sample time series and nonreducing gel format as that depicted in panel B. However, the sample shown is instead a Western blot probing the carboxy-terminal c-*myc* epitope of the 4-4-20-avitag protein.

except those listed later: anti-c-*myc* antibody, 9E10, was purchased from Covance (San Diego, CA), anti-HA antibody 12CA5, was purchased from Roche (Indianapolis, IN), AlexaFluor488-conjugated antimouse IgG, AlexaFluor555-conjugated antimouse IgG, and SA-Quantum dot 625 were purchased from Life Technologies (Grand Island, NY). Basic Fibroblast Growth Factor (bFGF) was purchased from Roche Diagnostics (Indianapolis, IN). PBSCM refers to phosphate buffer solution (PBS, 150 mM NaCl, 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4) supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgSO_4$. PBSCMG refers to PBSCM supplemented with 40% Goat Serum.

Strains, plasmids, and media

The plasmids pRS314-GAL-scFvA-Avitag or pRS314-GAL-4-4-20-Avitag were created from pRS316-GAL-scFvA[4] and pRS314-GAL-4-4-20[24] by appending an Avitag (GLNDIFEAQKIEWHE) near the carboxy-terminus (Figure 1A-i). ScFv secretion was performed using the *Saccharomyces cerevisiae* strain YVH10, which overexpresses protein disulfide isomerase.[25] Yeast were cultured in minimal SD-SCAA medium (10.19 g/L $Na_2HPO_4{\cdot}7H_2O$, 8.56 g/L $NaH_2PO_4{\cdot}H_2O$, 20 g/L dextrose, and 6.7 g/L yeast nitrogen base) supplemented with 2X SCAA amino acids (190 mg/L Arg, 108 mg/L Met, 52 mg/L Tyr, 290 mg/L Ile, 440 mg/L Lys, 200 Phe, 1260 mg/L Glu, 400 mg/L Asp, 480 mg/L Val, 220 mg/L Thr, and 130 mg/L Gly) with 40 mg/L tryptophan and 40 mg/L uracil at 30°C for 72 h in 1 L flasks. Subsequently, scFv secretion was induced by changing the culture medium from SD-SCAA to an equal volume of SG-SCAA (dextrose substituted by 20 g/L galactose) with 1 mg/mL BSA carrier, and incubating at 20°C for 72 h. The supernatants containing unfused scFv or Avitag-fused scFv were concentrated by Amicon Stirred Cells with ultrafiltration Discs (10 kDa) (Millipore, NH). The scFv or scFv-Avitag in the concentrated supernatant was then purified by Ni-NTA column (QIAGEN, Valencia, CA) as previously described[26] and dialyzed against 10 mM Tris-HCl, pH 8.0 buffer. The protein concentration was determined by resolving purified fractions by SDS-PAGE, along with BSA as a protein standard. After Coomassie blue staining of the resultant gel, the scFv protein concentrations were estimated by densitometry using Image J software.

The BirA enzyme was displayed on the yeast cell surface as previously described using the BirA-encoding plasmid pCT302-BirA (Kind gift from Dr. Eric Boder, University of Tennessee) and the EBY100 yeast surface display strain.[23] The yeast harboring the BirA display plasmid were grown in SD-CAA medium (10.19 g/L $Na_2HPO_4{\cdot}7H_2O$, 8.56 g/L $NaH_2PO4{\cdot}H_2O$, 5 g/L casamino acids, 6.7 g/L yeast nitrogen base, and 20 g/L dextrose) at 30°C overnight and induced in SG-CAA medium (dextrose substituted by 20 g/L galactose) at 20°C for 18 h. As the yeast surface display of BirA should also lead to display of flanking HA and c-*myc* tags, BirA display was confirmed by immunolabeling with mouse anti-HA antibody (clone 12CA5, 1:100 dilution) or anti-c*myc* clone 9E10 (1:100) followed by R-Phycoerythrin-conjugated goat antimouse secondary and analysis by flow cytometry.[23]

Biotinylation of scFvA-Avitag and 4-4-20-Avitag

The biotinylation of scFv-Avitag was catalyzed by BirA expressed on the yeast surface. The biotinylation reaction

was performed as follows: 2×10^9 BirA displaying yeast, 700 μL scFvA-Avitag (1 mg/mL), 100 μL biomix A (10 × concentration: 0.5 M bicine buffer, pH 8.3), 100 μL biomix B (10 × concentration: 100 mM ATP, 100 mM MgOAc, and 500 μM d-biotin), and 100 μL of 500 μM d-biotin were mixed and incubated at 30°C with shaking for 8 h. At different reaction time points (0.5, 1, 2, 3, 4, 6, 8 h), 5 μl of reaction supernatant was removed and mixed with 15 μL of 1 mg/mL SA solution and incubated at room temperature for 30 min, followed by SDS-PAGE gel analysis (see later). At the end of the reaction, the mixture was centrifuged at 14,000 × g for 10 min to remove all the yeast and then dialyzed in PBS pH 7.4 to remove the free biotin from the biotinylated scFv-Avitag.

Tetramer preparation

To prepare scFv tetramers, the biotinylated scFv (0.8 mg/mL in PBS, 20 μM) was mixed with SA at different molecular ratios to prepare the scFv tetramer. SA was added stepwise to the biotinylated scFv solutions in the following manner: 10% of the SA was mixed with biotinylated scFv and incubated at room temperature for 15 min, followed by another 10% aliquot of SA, and so forth, until reaching the biotinylated scFv: SA molar ratios of 1:1, 2:1, 4:1, and 8:1. The final mixture was incubated at room temperature for an additional 30 min prior to loading onto size exclusion chromatography (SEC) column for separation. A similar approach was used to prepare the biotinylated scFv conjugates with SA-quantum dot 625 (SA-Q-dot 625). The only difference was the molar ratio between biotinylated scFv and SA-Q-dot 625 was instead 24:1.

Size Exclusion Chromatography

A size exclusion TSK-GEL G3000SW_{XL} column (Tosoh Bioscience LLC, Montgomeryville, PA) and a BioCAD 700E chromatography work station (PerSeptive Biosystems, MN) were used to separate the scFv tetramers. The running conditions were as follows: eluent: 100 mM PBS buffer (pH = 7.4), flow rate: 0.5 mL/min, UV detector: 280 nm. Eluate samples were collected at 1-min intervals and subjected to SDS-PAGE to determine the scFv-tetramer concentration in the eluent for immunocytochemistry and brain perfusion experiments.

Gel electrophoresis and western blotting

The biotinylation reaction samples were mixed with SDS-containing sample buffer either with reducing reagent and boiling for 3 min or without reducing reagent and without boiling prior to loading onto a 7.5% SDS-PAGE gel. The gel was stained with Coomassie blue, or the resolved protein was transferred to a nitrocellulose membrane for Western blotting. The membrane was blocked with 5% fat free dry milk in PBST, probed with primary antibody, 9E10 (1:3000 dilution) followed by horseradish peroxidase-conjugated antimouse IgG (1:2000 dilution). Membranes were subsequently developed with ECL reagents and exposed to Hyperfilm (GE Healthcare, Buckinghamshire, HP8 4SP, UK).

RBE4 cell culture

The rat brain endothelial cell line (RBE4) was a kind gift from Dr. Françoise Roux.[27] RBE4 cells were grown at 37°C with 5% CO_2, in 45% Alpha Minimum Essential Medium, 45% Ham's F10 medium, and 10% heat inactivated fetal bovine serum supplemented with 100 μg/mL streptomycin, 100 unit/mL penicillin G, 0.3 mg/mL geneticin, and 1 μg/L basic Fibroblast Growth Factor on rat tail collagen Type I-coated 24-well tissue culture plates.

RBE4 cell binding and internalization

The RBE4 cells were grown to roughly 90% confluency before they were used for internalization experiments. The cells were immunolabeled with scFvA-tetramer, 4-4-20-tetramer or biotinylated scFvA-Avitag monomer at 0.2 μM in PBSCMG at 4°C for 30 min, and then switched to 37°C for another 30 min to allow internalization. Subsequently, the RBE4 cells were incubated with 9E10 (1:100 dilution) at 4°C for 30 min and AlexaFluor555-conjugated antimouse IgG antibody (1:500 dilution) in PBSCMG at 4°C for 30 min to detect the scFv-tetramer on the RBE4 cell surface. Next, the RBE4 cells were incubated with 0.5% saponin at 4°C for 5 min to permeabilize the cell membrane, again followed by labeling with 9E10 (1:100 dilution) at 4°C for 30 min. Finally, AlexaFluor488-conjugated antimouse IgG antibody (1:500 dilution) was administered for 30 min at 4°C to label the scFv-tetramer that had reached the RBE4 cell cytoplasm (along with some residual cell surface labeling). For the biotinylated scFv conjugated with SA-Q-dot 625, RBE4 cells were labeled at 4°C for 30 min and then switched to 37°C for another 30 min to induce the internalization. Subsequently, the RBE4 cells were incubated with 9E10 (1:100 dilution) at 4°C for 30 min and AlexaFluor488-conjugated antimouse IgG (1:500 dilution) at 4°C for 30 min to detect the scFvA-biotin-Q-dot 625 on the RBE4 cell surface. The presence of Q-dot 625 in the RBE4 cytoplasm was detected directly by its intrinsic fluorescence emission at 625 nm. Between each immunolabeling step, three PBSCM washes were used to remove nonspecific binding to RBE4 cells. Finally, the immunolabeled cells were postfixed with 4% paraformaldehyde and examined using a fluorescence microscope (Olympus IX70, Japan).

Brain tissue section immunohistochemistry

To detect the binding of scFv tetramer to the brain microvasculature, 8 micron rat brain cryosections were used. The rat brain section was blocked and permeabilized with 0.2% (v/v) triton-X-100 in PBSCMG at 4°C for 30 min. The brain section was then incubated with 0.2 μM monomeric, biotinylated scFv-Avitag or scFv-tetramer in PBSCMG at 4°C for 60 min. ScFv binding was detected by 9E10 immunolabeling (1:100 dilution) at 4°C for 30 min, followed by AlexaFluor555-conjugated goat antimouse IgG (1:500 dilution) mixed with fluorescein-labeled *Griffonia simplicifolia* agglutinin (GSA-FITC) (1:100 dilution) at 4°C for 30 min. Between each immunolabeling step, three PBSCM washes were used to remove nonspecific binding to the brain sections. To detect the binding of scFvA-Q-dot 625 to rat brain capillary endothelial cells, the rat brain section was blocked and permeabilized as described earlier and then incubated with 10 nM scFvA-Q-dot 625 or Q-dot 625 in PBSCMG at 4°C for 60 min followed by GSA-FITC (1:100 dilution) labeling at 4°C for 30 min. There were three PBSCM washes after brain section labeling steps to remove the nonspecific binding. Finally, the immunolabeled tissue sections were postfixed with 4% paraformaldehyde and examined with a fluorescence microscope (Olympus IX70, Japan).

Tetramer distribution in rat brain

Sprague Dawley rats (200–250 g, male, Harlan) were anesthetized with ketamine (50 mg/kg) and xylazine

(8 mg/kg), and an angiocatheter was placed into the left ventricle. The rats were perfused with 100 mL perfusate (0.9% NaCl, 100 unit/mL heparin, and 0.4% $NaNO_2$) to remove the blood, followed by 100 mL of 100 nM SEC-purified scFv-tetramer along with FITC conjugated with lectin from *Lycopersicon esculentum* (LEA-FITC, 10 μg/mL). Finally, the vasculature was perfused with an additional 100 mL of perfusate to remove unbound tetramer and LEA-FITC from the vasculature. As the 4-4-20 tetramer will bind LEA-FITC as FITC is its natural ligand, the 4-4-20 tetramer was instead perfused without LEA-FITC and the sections poststained with LEA-FITC. The rat brains were removed and embedded in Tissue-Tek O.C.T. Compound for sectioning. The brain sections were blocked and permeabilized with 0.2% Triton X-100 in PBSCMG at 4°C for 30 min, immunolabeled with 9E10 (1:100 dilution) at 4°C for 30 min followed by AlexaFluor555-conjugated goat antimouse IgG (1:500 dilution) without LEA-FITC (scFvA-tetramer brain sections) or with LEA-FITC (1:100, for 4-4-20 tetramer brain sections) at 4°C for 30 min. After washing three times with PBSCM, sections were postfixed with 4% PFA for 10 min, and imaged.

Results and Discussion

Expression and biotinylation of scFv-Avitag fusions

While the Avitag/BirA-based system has been used quite extensively for site-specific biotinylation, Avitag-protein fusions have largely been produced using bacterial,[28] insect,[15] and mammalian systems.[29] As scFvA was isolated from a yeast surface display library, and has been secreted from yeast as an scFv,[4] it was expected that the scFvA-Avitag fusion could also be produced using yeast. However, Avitag use has been much more limited with yeast systems and has been used either in intracellular cytosol-based biotinylation schemes[30] or by biotinylation of Avitag-linked proteins while in the secretory pathway.[31,32] Thus, we first tested whether Avitag-scFv fusions could be efficiently secreted from yeast. Both scFvA and an irrelevant antifluorescein scFv, 4-4-20, were cloned with an Avitag near the carboxy-terminus followed by the His_6 epitope and c-*myc* tags for purification and detection purposes, respectively (Figure 1A-i). Both scFv-Avitag and 4-4-20-Avitag fusions could be secreted from yeast and expression levels compared to the unfused scFv were analyzed by SDS-PAGE. While scFvA-Avitag was produced at roughly the same levels as unfused scFvA (~4 mg/L), the 4-4-20-Avitag fusion exhibited markedly decreased expression (~0.8 mg/L) compared to unfused 4-4-20 (~3 mg/L). However, both scFv-Avitag fusions were expressed at sufficient levels to further test biotinylation and tetramer formation.

Prior to biotinylation, the scFv-Avitag fusions were purified from the culture supernatants using Ni-NTA resin. As the presence of sodium in the Ni-NTA elution buffer is detrimental to biotinylation by BirA, purified scFv eluates were first dialyzed against Tris-HCl for buffer exchange. The BirA enzyme was displayed on the yeast surface, and BirA expression was confirmed by flow cytometry analysis as described by Parthasarathy et al.[23] (Figure 1A-ii). BirA displayed on the yeast surface has been demonstrated to be a robust biotinylation catalyst and as it remains tethered to the yeast surface, it can be separated from the biotinylated scFvs simply by centrifugation.[23] The biotinylation reaction was initiated by mixing the purified scFv-Avitag protein with the BirA-displaying yeast. The reaction extent was monitored by extracting reaction aliquots as a function of time and mixing with SA to evaluate the formation of scFv-Avitag-SA multimers (Figure 1A-iii); and hence, indirectly monitoring the biotin appendage on the scFv-Avitag proteins. As the interaction between biotin and SA is stable under conditions of nonreducing SDS-PAGE as described by Humber et al.,[33] the disappearance of the scFv-Avitag band from the coomassie stained gel (or Western blot tracking the c-*myc* epitope) as a function of reaction time is indicative of the amount of biotinylated scFv-Avitag (Figures 1B, C). As expected, given the relative concentrations of SA, biotinylated scFv-Avitag, and free biotin mixed in this assay, the multimers formed were predominantly monomers [SA-(scFvA-Avitag)] at shorter times with lower levels of biotinylated scFv-Avitag present it the reaction mixture. Over the reaction time, this distribution evolved to a roughly 50-50 mix of monomers and dimers [SA-$(scFvA\text{-}Avitag)_2$] where full biotinylation of scFv-Avitag was observed (Figures 1B, C). Both scFvA-Avitag and 4-4-20-Avitag were completely biotinylated within 4 h, which is a similar rate to that reported earlier using the yeast surface display BirA system with bacterially produced Avitag proteins.[23]

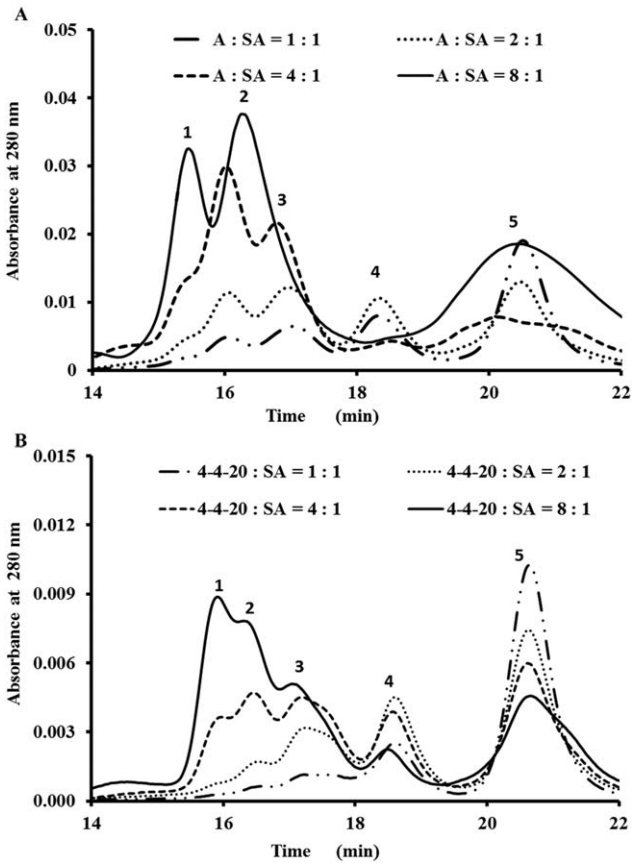

Figure 2. SEC analysis of tetramer formation.

(A) Tetramer formation using biotinylated scFvA-Avitag and (B) tetramer formation using biotinylated 4-4-20-Avitag. For both panels, SA was mixed stepwise with the biotinylated scFv to reach the final indicated molar ratios. Peaks indicated are: 1 = SA-$(scFv\text{-}Avitag)_4$, 2 = SA-$(scFv\text{-}Avitag)_3$, 3 = SA-$(scFv\text{-}Avitag)_2$, 4 = SA-(scFv-Avitag), 5 = scFv-Avitag.

Table 1. SEC Retention Time and Associated Molecular Weight of scFv Multimer Mixtures

Peaks	1	2	3	4	5
Biotinylated scFvA-Avitag:SA					
1:1	–	16.1	17.1	18.4	20.5
2:1	15.5	16.1	17.1	18.4	20.6
4:1	15.5	16.0	16.8	18.7	20.3
8:1	15.5	16.3	–	–	20.5
RT (min)[a]	15.5	16.1	17.0	18.5	20.5
SEC MW (kDa)[b]	278	219	153	85	39
Calculated MW (kDa)[c]	212(A_4S)	172(A_3S)	132(A_2S)	92(A_1S)	40(A_1)
Biotinylated 4-4-20-Aviatag:SA					
1:1	–	16.6	17.6	18.7	20.7
2:1	–	16.5	17.5	18.7	20.7
4:1	16.1	16.5	17.3	18.7	20.7
8:1	16.0	16.4	17.3	18.7	20.7
RT (min)[a]	16.1	16.5	17.4	18.7	20.7
SEC MW (kDa)[b]	219	187	132	79	37
Calculated MW (kDa)[c]	196(F_4S)	160(F_3S)	124(F_2S)	88(F_1S)	36(F_1)

[a]RT, average retention time of biotinylated scFv-Avitag tetramer, trimer, dimer, and monomer on SEC column.
[b]Molecular weight calculated based on the average retention time for each peak, based on molecular weight standards.
[c]Molecular weight based on amino acid sequence, MW (scFvA-Avitag, A) = 40 kDa, MW (4420-Avitag, F) = 36 kDa, and MW(streptavidin, S) = 52 kDa.

Preparation of scFv tetramers

As each SA can bind 0-4 biotinylated scFvs depending on the mode of mixing and relative concentration of the binding partners, it is optimal to add the SA stepwise to the biotinylated scFv-Avitag to keep molar excess of the scFv-Avitag such that there is a bias toward tetramer formation as opposed to lower level multimer formation.[15] In addition, to limit the amount of wasted, unconjugated scFv-Avitag, it would be desirable to have a final 4:1 molar ratio of biotinylated scFv-Avitag to SA given the 4 biotin-binding sites per SA molecule (Figure 1A-iii). Thus, for tetramer formation, SA was mixed by stepwise addition with scFvA-biotin and 4-4-20-biotin at differing molar ratios (see section Materials and Methods for details). The composition of the mixture in terms of the various multimer isoforms produced was evaluated by SEC (Figure 2). Based on the column retention times of protein molecular weight standards, it was possible to resolve chromatographic peaks approximately corresponding to the calculated molecular weight of SA conjugated to one (monomer, ~80 kDa), two (dimer, ~120 kDa, 4-4-20 only), three (trimer, ~160 kDa), or the desired four biotinylated scFv-Avitag molecules (tetramer, ~200 kDa) (Figure 2 and Table 1). As indicated in the chromatographic traces at the differing biotinylated scFv-Avitag: SA ratios, an 8:1 molar ratio of biotinylated scFv-Avitag to SA yielded the optimum percentage of tetramer formation (Figures 2A, B, Peak 1). However, while driving the mixture toward tetramer with some level of excess biotinylated scFv-Avitag (8:1) was helpful, there still remained a substantial portion of the biotinylated scFv-Avitag that was contained in other multimeric forms (Figure 2A, Peak 2 and Figure 2B, Peaks 2, 3, and 4). The first half of tetramer Peak 1 exiting the SEC column was collected for each scFv. While these tetramer preparations likely contained some trimer due to partial overlap with Peak 2, the recovered fractions should be predominantly comprised of tetramer. These "tetramer" preparations were

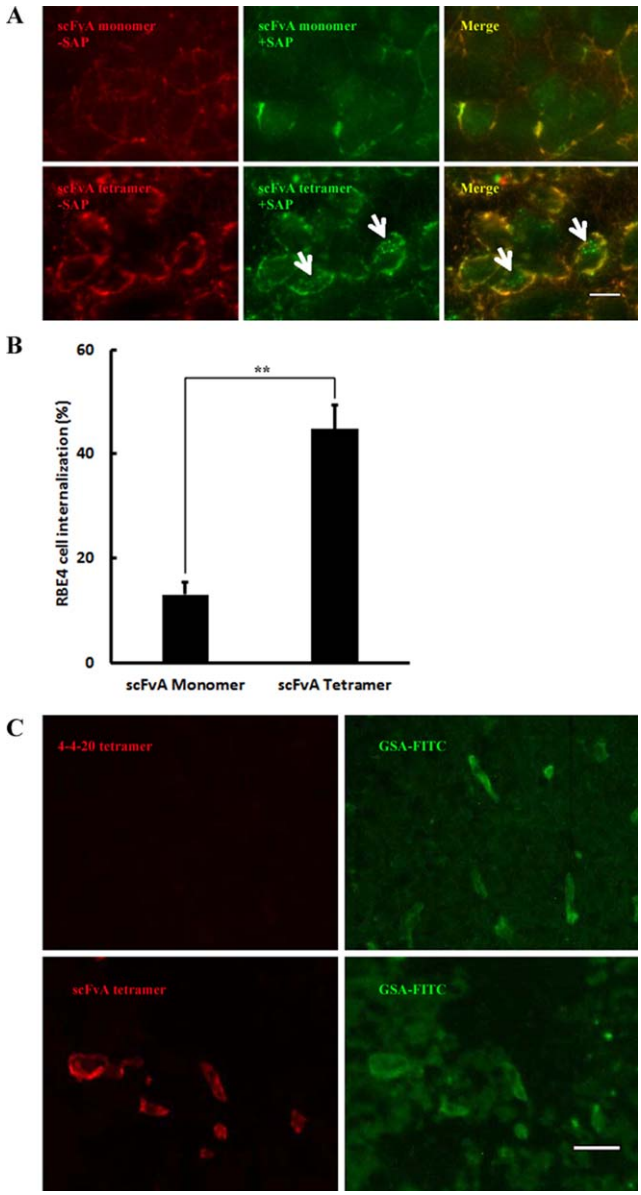

Figure 3. Evaluation of scFvA-tetramer binding and internalization.

(A) RBE4 cells were immunolabeled with biotinylated scFvA-Avitag or scFvA-tetramer at 4°C for surface binding and then switched to 37°C to allow internalization. Based on sequential fluorophore immunodetection, antibody binding to the cell surface in the absence of saponin-mediated cell permeabilization (-SAP) is depicted in red. Antibody localization determined after saponin-mediated permeabilization (+SAP) is indicated in green. Green punctate internalized structures that appear only after cell permeabilization are highlighted by white arrowheads. (B) Percentage of RBE4 cells having two or more punctate antibody-containing internalized vesicles. Three different immunocytochemistry fields of at least 50 cells each were quantified, $P < 0.001$ by unpaired Student's *t*-test. (C) Rat brain tissue sections were immunolabeled with scFvA-tetramer or 4-4-20-tetramer (red) and the vascular marker, GSA-FITC (green). Scale bars: 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

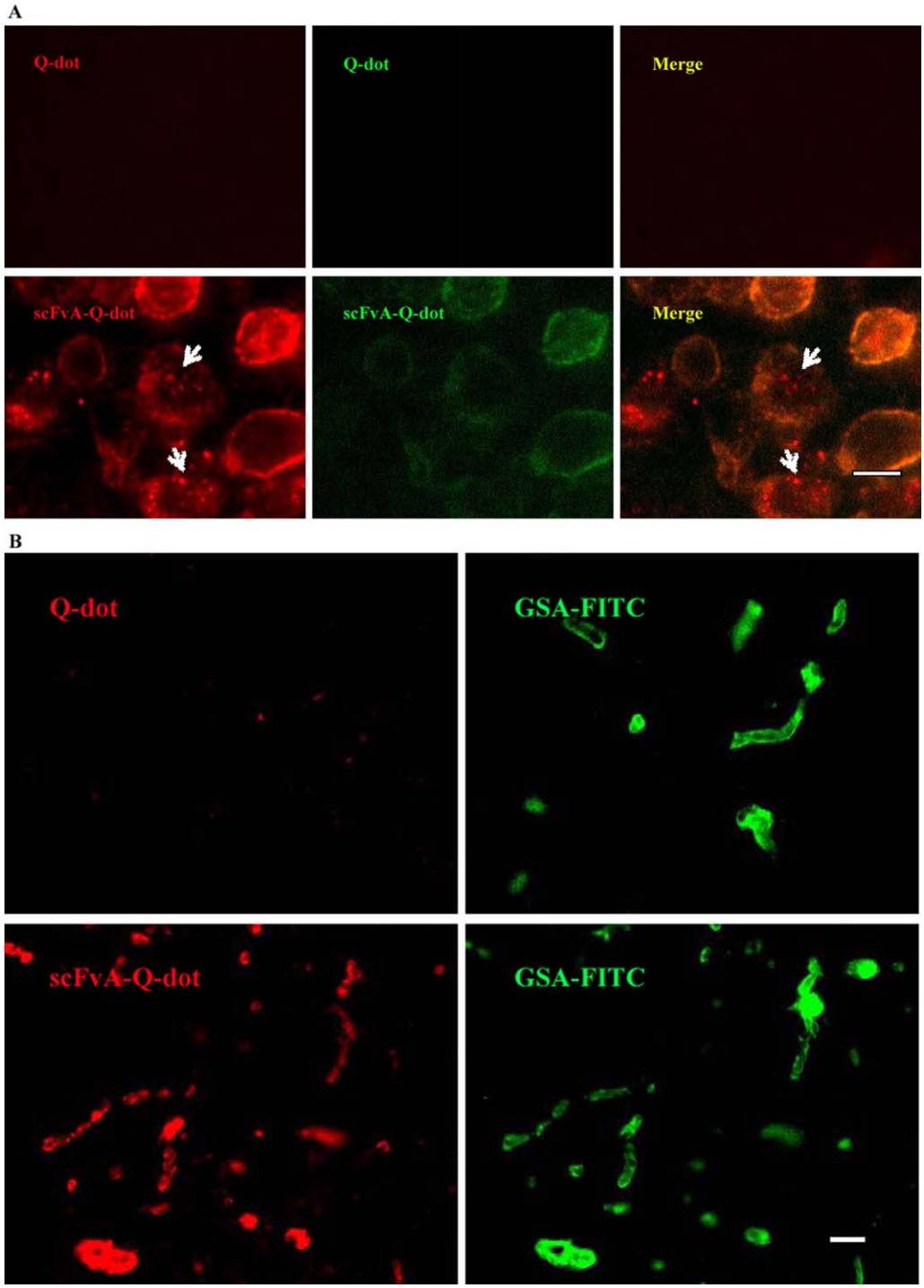

Figure 4. Evaluation of scFv-A-Q-dot 625 binding and internalization.

(A) RBE4 cells were immunolabeled with scFvA-Q-dot 625 or untargeted Q-dot 625 at 4°C for surface binding and then switched to 37°C to allow internalization. The unpermeabilized cells were then immunolabeled for detection of cell surface scFvA-Q-dot 625 (green). The white arrows indicate the internalized scFvA-Q-dot 625 (red). (B) Rat brain tissue sections were labeled with scFvA-Q-dot 625 or untargeted Q-dot 625 (red) and the vascular marker, GSA-FITC (green). Scale bar: 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

subsequently used for the characterization experiments below dealing with brain endothelial cell binding and internalization.

Binding and internalization into rat brain endothelial cells

Oftentimes it is possible to enhance cellular internalization by antibody multimerization that can drive clustering of cell surface targets.[34] Thus, internalization assays were performed using the RBE4 rat brain endothelial cell line and the internalization of biotinylated scFvA-Avitag was compared to that of SEC-purified scFvA-tetramers. Indeed, when the biotinylated scFvA-Avitag was used in the internalization assay, predominantly cell surface binding was detected with minimal internalization (Figures 3A, B). By contrast, the scFvA-tetramer mediated enhanced cell surface binding

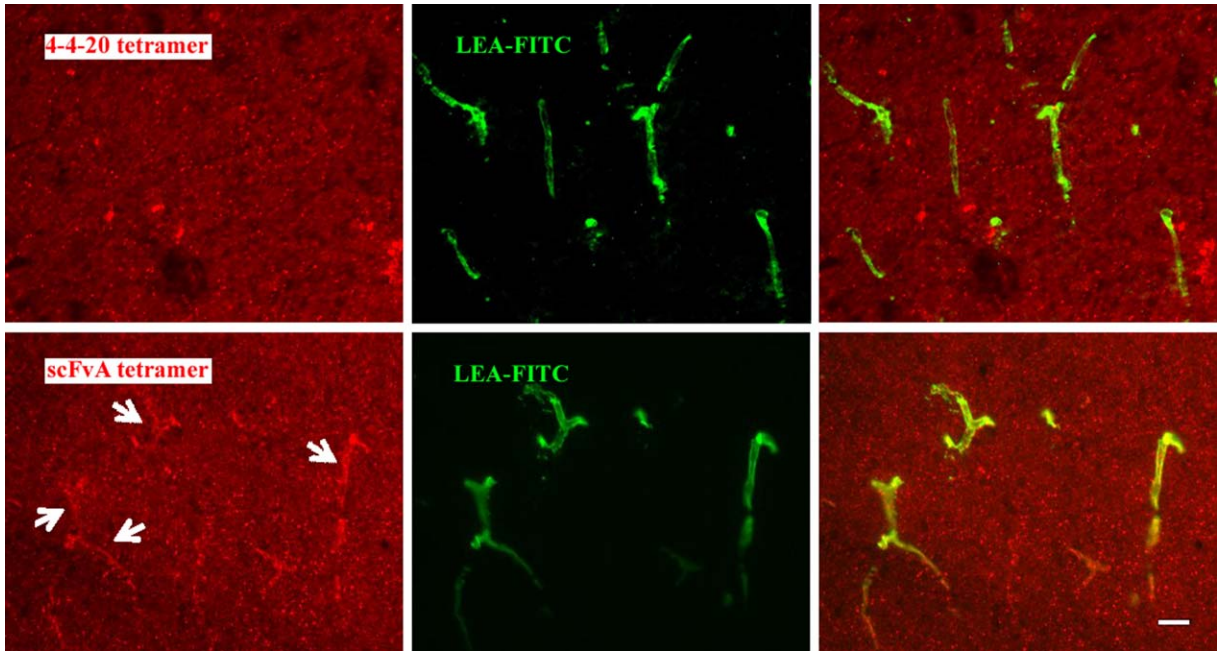

Figure 5. Tetramer distribution in rat brain.

Rats were perfused with scFvA-tetramer + LEA-FITC or 4-4-20-tetramer, respectively. Following an extensive perfusion of wash buffer to remove unbound tetramer, the brains were removed and tetramer distribution assessed. ScFv-tetramer (red) is shown along with the vascular marker LEA-FITC (green). Blood vessels having both scFvA-tetramer and lectin labeling are indicated by arrows. As 4-4-20 binds FITC as its natural antigen, the 4-4-20-tetramer brains were not perfused with LEA-FITC, but instead LEA-FITC was used to postlabel tissue sections. Scale bar: 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

compared to scFvA-Avitag alone along with substantially increased internalization (Figures 3A, B, $45 \pm 5\%$ vs. $13 \pm 2\%$ internalized, $P < 0.001$). Previously, it was shown that the unmodified scFvA could bind to brain capillaries in rat brain tissue sections in standard scFv format.[4] Thus, the binding of scFvA-tetramer to tissue sections was tested to ensure maintenance of brain capillary binding in the tetramer format. The binding of scFvA-tetramer on rat brain sections was completely colocalized with the endothelial cell marker fluorescein-labeled *Griffonia simplicifolia* agglutinin (GSA-FITC) as expected (Figure 3B).

Although the scFvA-tetramer could be internalized, we also wished to investigate if scFvA could act as a targeting molecule that could promote internalization of larger particles such as those that could be used in drug delivery strategies. As a reasonable facsimile, a quantum dot system was used because not only do quantum dots carry their own intrinsic fluorescence, they are also 15–20 nm particles.[35,36] In particular, a SA-coated quantum dot that fluoresces at 625 nm (SA-Q-dot 625) was investigated. This Q-dot possesses 6–8 SA per particle with 2–3 biotin-binding sites of each SA available. Thus, based on the experience forming scFvA-tetramers, a 24:1 molar ratio of biotinylated scFvA-Avitag to SA-Q-dot 625 was used to prepare scFvA-targeted Q-dots 625. Using the same internalization assay as described earlier for the scFvA-tetramers, the scFvA-Q-dot 625 conjugates were detected binding to the RBE4 cell surface and the scFvA-Q-dot 625 conjugates were also internalized into the RBE4 cells as indicated by the direct readout of 625 nm fluorescence (Figure 4A). In contrast, an untargeted Q-dot 625 did not bind or internalize into RBE4 cells, indicating that scFvA mediates the delivery of Q-dots 625 into the brain endothelial cells. The scFvA-Q-dots 625 also clearly immunolabeled brain capillaries in rat brain tissue sections (Figure 4B). Taken together, biotinylated scFvA-Avitag can mediate internalization into brain endothelial cells in tetrameric form or be used to target a Q-dot particle for internalization, and these promising results indicate the potential application of scFvA for delivery of therapeutic molecules or particles to brain endothelial cells.

Distribution in rat brain

Although binding and internalization are keys to any brain drug delivery paradigm, the capability of the scFvA-tetramers to bind to the blood side of the BBB *in vivo* is also a critical parameter. Therefore, the scFvA-tetramer was used to evaluate if the targeted antigen was expressed on the blood-facing plasma membranes of BBB endothelial cells *in vivo*. ScFvA-tetramer or negative control 4-4-20-tetramer were introduced into the rat brain via transcardiac perfusion techniques that allow the perfusate containing the scFv tetramers direct access to the brain vasculature. A FITC-conjugated lectin from *Lycopersicon esculentum* (LEA-FITC) was coperfused with scFvA-tetramers as a vascular marker.[37] Subsequently, the unbound antibody was washed out of the brain vasculature by saline perfusion. As indicated in Figure 5, binding of scFvA-tetramer was detected in brain capillaries and was colocalized with the LEA-FITC vascular perfusion marker. In contrast, 4-4-20-tetramer was not detected in postperfusion brains (Figure 5). These data indicate that the antigen targeted by scFvA-tetramer is accessible from the bloodstream, a key to any future *in vivo* application. In conclusion, we demonstrated that yeast can be used to both produce and biotinylate Avitag-coupled scFvs with reasonable yields. These biotinylated scFv could be formatted as tetramers or conjugated to Q-dots that promote brain

endothelial cell internalization *in vitro* and binding of the brain vasculature *in vivo*. As such, scFvA holds substantial promise as a brain drug targeting and delivery tool, pending detailed pharmacokinetic and biodistribution analyses.

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