Legomedicine—A Versatile Chemo-Enzymatic Approach for the Preparation of Targeted Dual-Labeled Llama Antibody–Nanoparticle Conjugates

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Supporting Information

ABSTRACT: Conjugation of llama single domain antibody fragments (Variable Heavy chain domains of Heavy chain antibodies, VHHs) to diagnostic or therapeutic nanoparticles, peptides, proteins, or drugs offers many opportunities for optimized targeted cancer treatment. Currently, mostly nonspecific conjugation strategies or genetic fusions are used that may compromise VHH functionality. In this paper we present a versatile modular approach for bioorthogonal VHH modification and conjugation. First, sortase A mediated transPEGylation is used for introduction of a chemical click moiety. The resulting clickable VHHs are then used for conjugation to other groups employing the Cu⁺-independent strain-promoted alkyne–azide cycloaddition (SPAAC) reaction. Using this approach, tail-to-tail bispecific VHHs and VHH-targeted nanoparticles are generated without affecting VHH functionality. Furthermore, this approach allows the bioconjugation of multiple moieties to VHHs for simple and convenient production of VHH-based theranostics.

INTRODUCTION

The challenge in cancer therapy is to specifically deliver therapeutic agents to tumor cells with minimal delivery to and effects on healthy tissues. Targeted delivery of drugs with antibody drug conjugates (ADCs) has received a lot of attention in the last decades. Also, nanoparticles have been used for drug delivery. Liposomes can carry hydrophilic drugs in the lumen, while micelles are suited for carrying hydrophobic drugs. Liposomes and micelles, however, distribute relatively randomly in the body after intravenous injection, and introduction of tumor specificity in these nanoparticles could greatly increase local delivery and efficacy of cancer therapeutics.

A number of therapeutic antibodies with relative tumor specificity are now applied clinically (e.g., trastuzumab and cetuximab against HER2 and EGFR). Effects of treatments with these targeted drugs are often limited because of recurrences of drug-resistant tumors. The challenge should therefore be to develop a multispecific tumor-targeting nanoparticle platform that can deliver cytotoxic payload to all cancer cells in a tumor, resulting in specific and acute death of all cells in a tumor.
Conventional protein conjugation strategies mostly use relatively nonspecific methods, e.g., N-hydroxysuccinimide (NHS) chemistry utilizing ε-NH₂ groups from lysines. Depending on the number and distribution of lysines in the active binding domain of the targeting agent, these conjugation protocols may compromise functionality. This specifically applies to single domain antibody fragments (VHHs, Nanobodies) due to their small size. VHHs are recombinant antigen-binding domains that are derived from camelid heavy chain-only antibodies and receive increasing interest as therapeutic or diagnostic compounds. \(^{13,14}\) VHHs typically have a molecular weight of 15–20 kDa and may bind target antigens with pM to nM affinity, similar to conventional antibodies. The ease of genetic engineering and handling of VHHs, combined with a number of other advantages such as high water solubility, low production cost, small size, low immunogenicity in humans, and high thermo- and pH stability, \(^{13}\) makes this class of antibodies a highly interesting alternative to conventional antibodies.

A number of VHHs with high specificity and affinity against tumor targets (e.g., EGFR, HER2, MET) have been developed. \(^{16−18}\) Approaches to conjugate VHHs without compromising functionality include the site specific introduction of a carboxyterminal cysteine allowing maleimide chemistry and introduction of a carboxyterminal five-amino-acid sequence (LPXTG) allowing sortase A transpeptidation. \(^{20}\)

Here we present a versatile modular approach for bioorthogonal VHH conjugations, using sortase A mediated transPEGylation to introduce a carboxyterminal click moiety, and subsequent Cu⁺-independent strain-promoted alkyne−azide cycloaddition (SPAAC) for conjugation of a functional group that may be connected to another VHH and/or polymeric micelles. Furthermore, introduction of a cysteine before the LPXTG tag allows maleimide chemistry to introduce a second diagnostic or therapeutic compound. This method allows the production of highly uniform VHH-based conjugates that can consist of multiple modular moieties, examples being labeled bispecific VHHs, bivalent VHHs, and VHH-targeted nanoparticles. Because all chemistry occurs at the carboxyterminus, this method does not affect VHH functionality. Furthermore, concomitant with conjugation, carboxy-terminal tags that allow purification of the VHHs after bacterial expression are removed, a prerequisite for future clinical applications.

### RESULTS AND DISCUSSION

VHHs were successfully expressed and purified as VHH-C-LPETG-8xHis-Vsv or VHH-LPETG-8xHis-Vsv fusion proteins with yields of 5–10 mg/L E. coli culture (see Figure 1A for a...
representative Coomassie Brilliant Blue (CBB) stained SDS-PAGE gel of 7D12-C-LPETG-8xHis-Vsv, the anti-EGFR VHH that was used as a prototype in this study). Since the 8xHis-Vsv tags are substituted by compounds of interest during the sortase A reaction, loss of these tags was compensated for by introducing a cysteine residue directly upstream of the LPETG-8xHis-Vsv sequence, allowing maleimide-based labeling with alternative tags for detection. Liquid chromatography mass spectrometry (LC-MS) indicated the correct mass for expressed 7D12-C-LPETG-8xHis-Vsv and it showed that the free thiol group of this cysteine was oxidized, presumably by glutathione based on its molecular weight of 307 Da (Figure 1B). After a mild TCEP reduction, the thiol was available for conjugation (Figure 1C).

The LPETG-8xHis-Vsv tag in 7D12[Fluo] allows sortase A mediated transpeptidation which releases the G-8xHis-Vsv tag in exchange for an H2N-GGG-containing peptide (the prototypical substrate of sortase A). This allows rapid and easy purification of the reaction product to homogeneity, because the G-8xHis-Vsv cleavage product and the 6xHis-tagged sortase A enzyme can be removed from the reaction mixture by Ni-bead depletion. Because a wide variety of chemically modified monodisperse PEG compounds is nowadays available, sortase A mediated conjugation of such compounds to the carboxyterminus is a highly attractive approach.

Using H2N-PEGx-X as a nucleophile for sortase A mediated PEGylation, a range of VHH-PEGX-X constructs can be generated in which X represents a drug or a reactive group. In this work we used the combination of H2N-PEG3-N3 and H2N-PEG3-DBCO in the SPAAC reaction.22 In an attempt to improve the reaction with H2N-PEGx-X, we tested recently described mutants of sortase A with optimized LPETG cleavage activity,23 and compared them to the variants with a N-terminal deletion of either 25 or 59 amino acids (Supporting Information Figure S8). These optimizations were performed using the anti-PlexinD1 VHH A12.24 Sortase A Δ59 was ultimately selected as most optimal, since despite increased LPETG cleavage rates, the low affinity of the mutant sortases for H2N-GGG or H2N-PEGx-X resulted in high levels of hydrolysis at the threonine.

Furthermore, we found that the sortase A ΔΔ59 variant, that contains an N-terminal 6xHis-tag, experienced unexplained proteolysis just downstream of the His-tag during the sortase A reaction (Supporting Information Figure S8D). Since this precluded complete removal of sortase A activity, we discarded this variant.

Figure 2. (A) Fluorescent image and (B) CBB staining of an SDS-PAGE gel of 7D12-C-LPETG-8xHis-Vsv, the anti-EGFR VHH that was used as a prototype in this study. Since the 8xHis-Vsv tags are substituted by compounds of interest during the sortase A reaction, loss of these tags was compensated for by introducing a cysteine residue directly upstream of the LPETG-8xHis-Vsv sequence, allowing maleimide-based labeling with alternative tags for detection. Liquid chromatography mass spectrometry (LC-MS) indicated the correct mass for expressed 7D12-C-LPETG-8xHis-Vsv and it showed that the free thiol group of this cysteine was oxidized, presumably by glutathione based on its molecular weight of 307 Da (Figure 1B). After a mild TCEP reduction, the thiol was available for conjugation (Figure 1C). The reaction of the 7D12-C-LPETG-8xHis-Vsv protein (from now on referred to as 7D12) with fluorescein-5-maleimide was efficient, resulting in a pure preparation of 7D12-C-[Fluo],LPETG-8xHis-Vsv (from now on referred to as 7D12[Fluo]) (Figure 2A+B, lane 1). LC-MS confirmed the conjugation of one fluorescein residue per VHH in 7D12[Fluo] (Figure 2C), demonstrating that the two native framework cysteine residues involved in an intramolecular disulfide bridge were not reactive toward fluorescein-5-maleimide under the conditions used. This was further confirmed in additional experiments that showed an absence of maleimide reaction with multiple VHHS lacking the C terminal cysteine (Figure S1).

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Further experimentation using different molar ratios of H2N-PEG2-X or H2N-GGG revealed that H2N-PEG2-X was incorporated less efficiently than H2N-GGG, requiring a 200-fold molar excess of H2N-PEG2-X over VHH-(C)LPETG-8xHis-Vsv as compared to a 25-fold excess of H2N-GGG (under conditions of 4 h reaction at 30 °C with sortase A Δ59). Lower concentrations of both nucleophiles in the reaction resulted in the formation of VHH-LPET-OH hydrolysis products, as observed with LC-MS (Supporting Information Figure S9A). These results were in line with those of Parthasarathy et al., who showed that conjugation of H2N-PEG2 to EGFP in a ratio of 1:1 by sortase A Δ59 was highly inefficient.25 Yet, the use of H2N-PEG2, X nucleophiles instead of triglycine-containing substrates would have an important advantage: conjugation of H2N-PEG2-X yields VHH-(C)LPET-PETG-X in a one-way reaction, unlike conjugation of H2N-GGG-X that reconstitutes a sortase A N-substrate site in the VHH-(C)LPET-GGG-X reaction product. Indeed we could show that VHH-LPET-PEG2-X is not a substrate for sortase A, in contrast to VHH-LPETGGG-peptide (see Figure S2).

Using the optimized conditions, 7D12[Fluo] was conjugated to H2N-PEG2, N5 with sortase A Δ59 to yield 7D12[Fluo],N1 (Figure 2A+B). After removal of sortase A Δ59, G-8xHis-Vsv, and unreacted 7D12[Fluo] by Ni-NTA depletion, 7D12[Fluo],N1 was obtained in a 66% yield with a >95% purity based on LC-MS (Figure 2D). Similar results were obtained when H2N-PEG2, DBCO was used in the reaction with sortase A Δ59 (Figure S3). Furthermore, the methodology has been successfully tested and validated for three more VHHs in our lab (Supporting Information Figure S10) demonstrating the versatility of this approach.

Bispecific or bivalent antibody conjugates are valuable compounds for targeted cancer therapy. Current approaches to make bispecific or bivalent VHHs often involve genetic fusion of VHH open reading frames, allowing production only in head-to-tail fusion format. It has already been shown that the affinity of the second VHH in such bispecific constructs may be dramatically affected by the presence of the first VHH.25 Sortase A technology allows the generation of tail-to-tail dimers, leaving antigen binding sites of the individual composing VHHs intact (Figures 3 and 4A). We tested the validity of this hypothesis by

![Diagram](image)

Figure 3. Schematic representation of the monomeric VHH, head-to-tail bispecific VHH, and tail-to-tail bispecific VHH and their nomenclature in this report.

using sortase A Δ59 in combination with H2N-PEG2, X linkers to generate 7D12[Fluo],N3 and 4E4-DBCO, 4E4 being a low-affinity Transferrin receptor (TRK)-binding VHH (unpublished results) that is used here as a control. After the click reaction (see Figure 4B+C for analysis of the reaction products), heterodimer N7D12[Fluo]-4E4 could be readily purified from residual monomers by size exclusion chromatography, resulting in >90% pure product of 36 kDa (Figure S4). In parallel, we generated conventional head-to-tail bispecific VHHs, with 7D12 and 4E4 separated by a [G4S], linker or a [G4S], linker and a C-terminal C-Flag-6xHis (compounds N7D12[Fluo]-[G4S],NE4[Fluo] and N4E4[Fluo]-[G4S],N7D12[Fluo]; Figures 3 and 4A). E. coli expression of these bispecific VHHs was successful as shown with SDS-PAGE gel. 1 = N7D12[Fluo]-[G4S],NE4[Fluo], 2 = N4E4[Fluo]-[G4S],N7D12[Fluo], 3 = N7D12[Fluo]-[G4S],N7D12[Fluo], 4 = N4E4[Fluo]-[G4S],N7D12[Fluo]. (F) Flow cytometry histogram derived from A431 cells incubated with the various bispecific constructs and controls.

![Flow Cytometry](image)

Figure 4. (A) Schematic overview of bispecific tail-to-tail (Structure 1) or head-to-tail (Structure 2) VHH constructs. In panels B and C, SDS-PAGE is shown (fluorescence signal and CBB staining, respectively) depicting the products of the sortase A reaction to produce bispecific 7D12[Fluo]-4E4, 1 = 7D12[Fluo], 2 = 4E4, 3 = 7D12[Fluo], DBCO, 4 = 4E4-N5, 5 = N7D12[Fluo]-4E4[Fluo] before sephadex G75 purification. In panels D and E, the expression of bispecific head-to-tail VHHs is depicted with (D) fluorescence signal and (E) CBB staining of an SDS-PAGE gel. 1 = N7D12[Fluo]-[G4S],NE4[Fluo], 2 = N4E4[Fluo]-[G4S],N7D12[Fluo], 3 = N7D12[Fluo]-[G4S],N7D12[Fluo], 4 = N4E4[Fluo]-[G4S],N7D12[Fluo]. (F) Flow cytometry histogram derived from A431 cells incubated with the various bispecific constructs and controls.

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Figure 5. Chemical structures and corresponding $^1$H NMR-spectra of (A) Ben-PCL$_7$-mPEG$_{2000}$ and (B) Ben-PCL$_7$-PEG$_{2000}$-DBCO measured in CDCl$_3$.

Figure 6. (A) Cartoon of VHH$^{[\text{Fluo}]}$-decorated mTHPC loaded micelles. (B) Fluorescein visualization. (C) CBB staining of the SDS-PAGE analysis of SPAAC reaction between 7D12$^{[\text{Fluo}]}$-N$_3$ and DBCO-micelles. 1 = 7D12$^{[\text{Fluo}]}$, 2 = 7D12$^{[\text{Fluo}]}$-N$_3$, 3 = 10% DBCO-micelles with 5% mTHPC, and 4 = 10% DBCO-micelles with 5% mTHPC and 5% 7D12$^{[\text{Fluo}]}$. (D) Median values of fluorescence intensity as measured in flow cytometry. Left bar graph = fluorescein total signal on A431 (EGFR$^+$) and E98 (EGFR$^-$) cells after incubation with 7D12$^{[\text{Fluo}]}$ as a positive control and 7D12$^{[\text{Fluo}]}$-micelles. Right bar graph = mTHPC total signal on both A431 and E98 cells after incubation with targeted or nontargeted micelles. * indicates significance with $p < 0.05$. (E) Fluorescence microscopy of A431 cells immediately after incubation with 1 = 7D12$^{[\text{Fluo}]}$, 2 = 7D12$^{[\text{Fluo}]}$-micelles, and 3 = nontargeted micelles. Cyan depicts mTHPC, incorporated in the micelles; green depicts fluorescein. The scale bar depicts 20 $\mu$M.
N7D12[Fluo], 1C-4E4[Fluo] were equally effective as 7D12[Fluo] in binding to A431 cells. In contrast, binding to EGFR was reduced with a factor of 10 for compounds N4E4-[G4S]2-ND712[Fluo] with both G4S linker lengths, confirming that, at least for VHH 7D12, epitope binding is hindered by the presence of a VHH at the amino-terminus. Thus, bispecific VHHs generated via tail-to-tail click fusion are more robust than head-to-tail bispecific VHHs.

PEGylation is an important modification of nanoparticles that is applied to increase half-life in the circulation by avoiding rapid clearance by spleen, liver, and kidney. 29 PEGylation of VHHs, accomplished by clicking DBCO-PEG to VHH-N3, indeed results in better in vivo characteristics. 30 Sortase A mediated conjugation of H2N-PEG3x yields the interesting option to decorate PEGylated nanoparticles with functionally active VHHs. To test this hypothesis we applied click chemistry to conjugate 7D12[Fluo]-N3 to benzoyl-poly(e-caprolactone)-methoxypoly(ethylene glycol) (ben-PCL7-mPEG2000) diblock-polymers, equipped with a small percentage of DBCO groups. Ben-PCL-mPEG2000 diblock-based micelles are bio-compatible and biodegradable, and can be used as carriers of hydrophobic drugs that can be incorporated in the micellar core. 31 The decoration of micelles with VHHs may increase affinity of the nanoparticles to targets by an avidity effect. Also, this approach allows the generation of multispecific drug-loaded targeting nanoparticles by simply decorating these with different tumor-targeting VHHs.

1H NMR spectra showed that synthesis of intermediate products (Figure S6) and end products ben-PCL-mPEG2000 and ben-PCL-mPEG2000-DBCO (Figure S5A+B) was successful. Micelles were prepared by film-hydration of a mixture of 90% ben-PCL-mPEG2000 diblock polymers and 10% ben-PCL-mPEG2000-DBCO, resulting in micelles that contain a calculated 20–30 DBCO click groups per particle. Dynamic light scattering (DLS) experiments of the resulting micelles revealed a mean particle size of 28 ± 2 nm and a polydispersity index (PDI) of 0.32 ± 0.02.

DBC0-micelles were decorated with 7D12 via a click chemistry reaction with 7D12[Fluo]-N3 in a 2:1 DBCO:N3 ratio, to minimize amounts of residual unconjugated 7D12[Fluo]-N3 after the click reaction. To confirm that during handling and experimentation micelles remained intact, we confirmed the hydrophobic photosensitizer meta-tetra(hydroxyphenyl)chlorin (mTHPC) during micelle formation, equipping the micelles with a fluorescent signal that is readily distinguishable from 7D12-associated fluorescein. SDS-PAGE of the 7D12 conjugated micelles revealed a shift of ~3 kDa as compared to the starting material 7D12[Fluo]-N3, indicating successful conjugation of 7D12[Fluo]-N3 to the DBCO-block-copolymer with a conjugation yield of >85% (Figure 6B,C). Again this conjugation method was verified for other available VHHs in our lab (Supporting Information, Figure S1A+B), showing its versatility.

We next tested whether 7D12 on the decorated micelles had retained EGFR affinity by performing flow cytometry after incubation with EGFR-positive A431 squamous cell carcinoma or EGFR-negative E98 glioma cells, 32 using 7D12[Fluo] as reference. 7D12[Fluo]-micelles showed efficient binding to A431 cells but negligible binding to E98 cells (Figure 6D). Importantly, binding of 7D12[Fluo]-micelles led to 70% higher cell-associated fluorescein-fluorescence than binding of monomeric 7D12[Fluo]. This may reflect the loading of individual micelles with multiple fluorescein-labeled 7D12 moieties or may be due to cooperative binding and an avidity effect. Increased binding of 7D12[Fluo]-micelles to A431 cells was confirmed with fluorescence microscopy, using the fluorescein signal as readout (Figure 6E, green signal in panel 2, compare to 7D12[Fluo] in panel 1). The increase in fluorescein signal was accompanied by an increased association of mTHPC fluorescence in 7D12[Fluo]-micelles as compared to nondecorated micelles (Figure 6D, right graph, and Figure 6E, cyan signal). There was no difference in binding of 7D12[Fluo]-micelles and nondecorated micelles to E98 cells, suggesting that 7D12[Fluo]-micelles bound as intact particles to A431 cells in an EGFR-dependent manner. It must however be noted that there was also some nonspecific association of mTHPC with both A431 cells and E98 cells. Whether this reflects nonspecific binding of micelles or of free mTHPC that is released from micelles is difficult to discriminate.

These results indicate that the multivalency induced by coupling multiple VHHs to a micelle, increased uptake of these particles and their payload in target positive cells. Although we restricted ourselves in this study to micelles, this conjugation approach is predicted to work with many other types of protein- or PEG-based nanoparticles (e.g., liposomes, polymersomes) once a controlled percentage of the building blocks is equipped with a chemical click group. Interestingly, this procedure will also allow the controlled synthesis of multispecific targeting nanoparticles, e.g., by preparing nanoparticles with a defined number of noncompatible clickable agents such as DBCO and tetrazine moieties, allowing simultaneous conjugation of VHH-PEG2-N3 and VHH-PEG2-TCO (trans-cyclooctene). Whereas the free thiol group in VHH-C-LPETG-PEG2-X may be used for maleimide-based conjugation of hydrophilic drugs, micelles may be used to simultaneously carry hydrophobic drugs, making this to a versatile approach.

**CONCLUSION**

Conjugations of VHHs to diagnostic or therapeutic compounds should involve the VHH’s carboxy-terminus, distant from the antigen binding site, in order to retain full VHH functionality. Here, a bioorthogonal site-specific conjugation approach is presented based on sortase A and click chemistry, combined with cysteine–maleimide conjugation. This approach allows the preparation of molecularly defined targeted nanoparticles, preserving targeting potential and concomitantly removing unwanted tags from VHHs, a prerequisite for potential clinical applications. The potential to perform controlled dual labeling of proteins without the loss of protein function is an important next step to the preparation of optimized theranostics.

**EXPERIMENTAL PROCEDURES**

**VHH Production and Purification.** VHH 4E4 is a low affinity VHH (Kd ~ 500 nM) directed against transferrin receptor (TfR) and was used as an inert VHH in this study. VHH 7D12 against EGFR has been described in detail before. 7,33 The VHH coding sequences were cloned in frame behind the pelB leader sequence in modified pHEN-IX vectors in which the sortase A recognition sequence LPETG, either or not preceded by a cysteine, was inserted just upstream of the 8xHis-Vsv tags, resulting in pHENIX-VHH-LPETG-8xHis-Vsv and pHENIX-VHH-C-LPETG-8xHis-Vsv. Plasmids were transformed in E. coli strain ER2566 for standard protein expression. Cells were grown in 2xTY medium containing 3.5% (w/v) glycerol and 50 μg/mL ampicillin at 37 °C. At an OD600 between 0.6 and 0.8, recombinant protein expression was induced with 1.0 mM...
isopropyl β-D-thiogalactoside (IPTG, Serva, Heidelberg, Germany) at 30 °C for 2.5 h. Cells were harvested by centrifugation at 2830 g for 20 min at 4 °C and the periplasmic protein fraction was isolated via osmotic lysis. Cells were resuspended in ice cold TES buffer (200 mM Tris pH 8.0, 0.5 mM EDTA, 20% w/v sucrose, protease inhibitors (Complete cocktail, Roche, Basel, Switzerland)) and incubated for 20 min on ice, followed by centrifugation (4424 g, 20 min, 4 °C). After collection of the supernatant the bacterial pellet was resuspended in TES buffer (4 °C). After washing of the beads with 50 mM phosphate pH 7.4, 500 mM NaCl, and 10 mM imidazole, 8xHis-tagged proteins were eluted with 500 mM imidazole in 50 mM phosphate pH 7.4 and 500 mM NaCl. The eluate was dialyzed against 50 mM TRIS pH 7.5 and 150 mM NaCl in a 3.5 kDa dialysis membrane (Spectrum laboratories, Los Angeles, CA, USA). VHHs were analyzed by SDS-PAGE under reducing conditions (Coomassie brilliant blue (CBB) staining), followed by analysis on the Odyssey CLx infrared imaging system (LI-COR, Lincoln, NE, USA) and liquid chromatography and mass spectrometry (LC-MS, Shimadzu HPLC and Thermo Finnigan LCQ Fleet) on a C4 column. Protein concentrations were determined by absorbance at 280 nm using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

**Generation and Production of In Tandem VHH Dimers.** In tandem bispecific N7D12C-N4E4C and the converse orientation N4E4C-N7D12C VHHs connected by [G4S]n-NVHHb linkers. The N-terminal VHH was amplified with 50 mM phosphate pH 7.4, 500 mM NaCl and 10 mM imidazole, 8xHis-tagged proteins were eluted with 500 mM imidazole in 50 mM phosphate pH 7.4 and 500 mM NaCl. The eluate was dialyzed against 50 mM TRIS pH 7.5 and 150 mM NaCl in a 3.5 kDa dialysis membrane (Spectrum laboratories, Los Angeles, CA, USA). VHHs were analyzed by SDS-PAGE under reducing conditions (Coomassie brilliant blue (CBB) staining), followed by analysis on the Odyssey CLx infrared imaging system (LI-COR, Lincoln, NE, USA) and liquid chromatography and mass spectrometry (LC-MS, Shimadzu HPLC and Thermo Finnigan LCQ Fleet) on a C4 column. Protein concentrations were determined by absorbance at 280 nm using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

**Sortase A Mediated Conjugation of Click Moieties.** To produce clickable fluorescent VHHs, 50 μM VHH[Fluo] was incubated overnight at RT in the dark with 50 μM sortase A ΔS59 and 4.0 mM H2N-PEG-X-N3 or H2N-PEG-X-DBCO (Jena Biosciences, Jena, Germany) in 50 mM Tris pH 7.5, 150 mM NaCl supplemented with 10 mM CaCl2. This reaction induces the covalent linkage of PEG linkers via the amine group to the threonine in the LPETG tag, releasing G-8×His-Vsv. Sortase A ΔS59, the cleaved G-8×His-Vsv tag from the reduced VHH, and residual intact VHH were removed by adsorption to Ni-NTA sepharose, pre-equilibrated with 50 mM phosphate pH 7.4 and 500 mM NaCl, for 1 h at 4 °C. Then, the excess of unreacted PEG linkers was removed by filtration in a 10 kDa MWCO centrifugal unit. The reaction product was washed three times with 50 mM phosphate pH 7.4 and 500 mM NaCl, and two times with 50 mM phosphate pH 7.4 and 500 mM NaCl containing 20% w/v glycerol. The protein constructs were analyzed by SDS-PAGE under reducing conditions and LC-MS, and protein concentration was determined by ultraviolet absorbance at 494 nm (ε494nm = 70 000 M⁻¹ cm⁻¹) using a Nanodrop spectrophotometer.

**Generation of VHH Dimers via C-to-C Conjugation.** To produce coupled bispecific VHHs were produced by incubating 4E4-N5 with 7D12[Fluo] in a 1:1 molar ratio at RT. Bispecific N7D12[Fluo]-4E4 was separated from single VHHs by separation on a G75 Sephadex (Pharmacia fine chemicals, Uppsala, Sweden) column. Fractions were analyzed on a SDS-PAGE gel under reducing conditions and LC-MS, and protein concentration was determined by ultraviolet absorbance at 494 nm.
Brieﬂy, Ben-PCL-OH macromers were prepared using benzyl alcohol as the initiator in the Tin(II) ethylhexanoate (Sn(Oct)2) catalyzed ring opening polymerization of ε-caprolactone (CL) (Figure 7A). The monomer to initiator ratio was chosen such that the average degree of polymerization was 7 CL units. The hydroxyl end groups of ben-PCL7-OH were subsequently reacted with p-nitrophenyl chloroformate to form p-nitrophenyl carbonate substituted polymers (PNC) (Figure 7B). The diblock copolymers were obtained by reacting ben-PCL7-PNC and mPEG2000-NH2 at 1:1 ratio in toluene at RT (Figure 7C).

Average molecular weights of the polymers were determined by NMR.

Ben-PCL7-PEG2000. NH2-PEG2000-NHBoc (1.00 g, 0.5 mmol) (Layson Bio, Arab, AL, USA) was added to a solution of ben-PCL7-PNC (0.707 g, 0.5 mmol) in 20 mL dry toluene. This mixture was stirred for 1 h at RT under nitrogen atmosphere (Figure 7D). The mixture was washed at least 6 times with diethyl ether to remove p-nitrophenol. The product was dried in a vacuum oven and obtained as a white powder (yield: 97%). Subsequently, 0.2 g of the powder was dissolved in 20 mL of DCM and the Boc group was removed by bubbling HCl gas through the solution for 15 min (Figure 7E). The product, ben-PCL7-PEG2000-NH2 (0.187 g, 0.063 mmol), was purified by precipitation in diethyl ether and subsequently dissolved in 20 mL of dry DCM. To produce clickable copolymers, dibenzocyclooctyne (DBCO)-NHS (0.025 g, 0.063 mmol) (Jena Biosciences, Jena, Germany) was added to this ben-PCL7-PEG2000-NH2 solution, and the reaction mixture was stirred for 24 h at RT under a nitrogen atmosphere (Figure 7F). The mixture was carefully washed with diethyl ether to remove unreacted DBCO-NHS. The product was dried in a vacuum oven and obtained as a white powder (yield: 61%).

1H (300 MHz) NMR spectra were recorded using a Gemini NMR spectrometer (Varian Associates Inc. NMR instruments, Palo Alto, CA). Polymers were dissolved in CDCl3 at a concentration of 0.015 g mL−1.

Dynamic Light Scattering. The size and the size distribution of empty particles were measured by dynamic light scattering (DLS) using a Malvern CGS-3 multangle goniometer (Malvern), consisting of a HeNe laser source (λ = 632.8 nm, 22 mW output power), temperature controller (Julabo water bath) and a digital correlator ALV-5000/EPP. Time correlation functions were analyzed using the ALV-60X0 Software v 3.X provided by Malvern, to obtain the Z-average hydrodynamic diameter of the particles (Zave) and the particle size distribution (polydispersity index, PDI). The samples were analyzed at 25 °C.

7D12 Functionalized mTHPC Loaded Micelle Preparation. Micelles were formed by film-hydration. To prepare micelles with 10% functional groups on the surface, ben-PCL7-PEG2000 and ben-PCL7-PEG2000-DBCO were dissolved in dichloromethane (DCM, Sigma-Aldrich, Saint Louis, MS, USA) and mixed in a 9:1 weight ratio in a 5 mL glass vial (VWR, Radnor, PA, USA). To this mixture, the hydrophobic photosensitizer mTHPC (meta-tetra(hydroxyphenyl) chloride, Biolitec AG, Jena, Germany) in tetrahydrofuran (THF, Sigma-
mTHPC-ben-PCL7-PEG2000 micelles were prepared according to conditions (Fluorescein signal and CBB staining). Nontargeted mTHPC-ben-PCL7-PEG2000 micelles were prepared according to the same procedure, but without incubation with 7D12[Fluo]-N3.

Cell Culture. The EGFR-overexpressing squamous carcinoma cell line A431 and the EGFR-negative glioma cell line E98 were cultured in DMEM (Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 40 μg/mL gentamycin (Centrafarm, Etten-Leur, The Netherlands). Cells were incubated at 37 °C with continuous shaking at 450 rpm. The final product was analyzed by SDS-PAGE under reducing conditions (Fluorescein signal and CBB staining). Nontargeted mTHPC-ben-PCL7-PEG2000 micelles were prepared according to the same procedure, but without incubation with 7D12[Fluo]-N3.

Flow Cytometry and Confocal Fluorescence Microscopy Analysis. Cells were dissociated from culture flasks with 10 mM EDTA, counted, and transferred to V-bottom-shaped 96-well microplates (BD Biosciences, Franklin Lakes, NJ, USA) at 5 × 10^5 cells/well. All subsequent steps were done on ice and all washing steps were executed by centrifugation of the plates at 1,500xg for 2 min. Cells were washed twice with PBS and aspecific binding sites were blocked by preincubating cells for 10 min with PBS (0.5% BSA, 2% FCS). Subsequently cells were incubated with 1 μM fluorescein-labeled VHHs in PBA for 20 min, washed and resuspended in PBA, and analyzed using the CyAn ADP analyzer (Beckman Coulter, Fullerton, CA, USA).

To analyze uptake of VHH-micelles with flow cytometry, cells were grown to 80% confluence in 8-well chambered glass slides (NUNC, Thermo Fisher Scientific, Waltham, MA, USA) and incubated with 20 μM (≈0.6 mg/mL) targeted or nontargeted mTHPC-micelles or equimolar VHH concentrations at 37 °C for 30 min. Cells were washed twice with warm DMEM and dissociated with trypsin at 37 °C. Then, cells were taken up in PBA and analyzed using the CyAn flow cytometer. mTHPC was quantified with parameter FL-8, fluorescein was quantified with parameter FL-1. To visualize binding and uptake with confocal microscopy A431 and E98 cells were grown in 8 wells Lab-Tek borosilicate coverglass chambers (Nunc, ThermoFisher Scientific, Waltham, MA, USA), and incubated with the micelles and controls as described for flow cytometry. After washing, cells were kept in phenol red free DMEM supplemented with 20 mM HEPES, and imaged on a TCS SP8 microscope (Leica Microsystems, Mannheim, Germany) equipped with a HC PL APO CS 40X/0.85 dry objective. During imaging, cells were maintained at 37 °C. The UV405 laser was used for excitation, and emission was collected between 500 and 600 nm for fluorescein and 630 and 730 nm for mTHPC.

**ASSOCIATED CONTENT**

*Supporting Information*

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.6b00638.


