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Facile Double-Functionalization of Designed Ankyrin Repeat Proteins using Click and Thiol Chemistries

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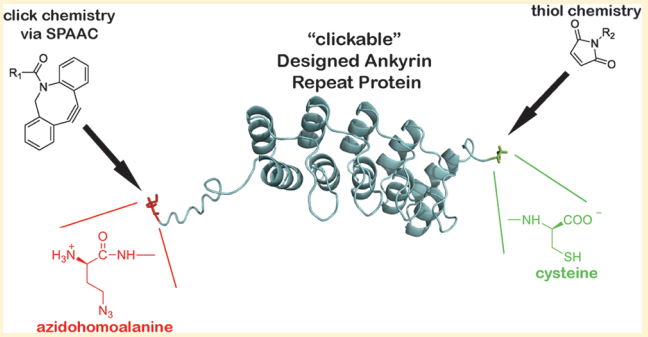
Supporting Information

ABSTRACT: Click chemistry is a powerful technology for the functionalization of therapeutic proteins with effector moieties, because of its potential for bio-orthogonal, regio-selective, and high-yielding conjugation under mild conditions. Designed Ankyrin Repeat Proteins (DARPins), a novel class of highly stable binding proteins, are particularly well suited for the introduction of clickable methionine surrogates such as azidohomoalanine (Aha) or homopropargylglycine (Hpg), since the DARPin scaffold can be made methionine-free by an M34L mutation in the N-cap which fully maintains the biophysical properties of the protein. A single N-terminal azidohomoalanine, replacing the initiator Met, is incorporated in high yield, and allows preparation of "clickable" DARPins at about 30 mg per liter *E. coli* culture, fully retaining stability, specificity, and affinity. For a second modification, we introduced a cysteine at the C-terminus. Such DARPins could be conveniently site-specifically linked to two moieties, polyethylene glycol (PEG) to the N-terminus and the fluorophore Alexa488 to the C-terminus. We present a DARPin selected against the epithelial cell adhesion molecule (EpCAM) with excellent properties for tumor targeting as an example. We used these doubly modified molecules to measure binding kinetics on tumor cells and found that PEGylation has no effect on dissociation rate, but slightly decreases the association rate and the maximal number of cell-bound DARPins, fully consistent with our previous model of PEG action obtained *in vitro*. Our data demonstrate the benefit of click chemistry for site-specific modification of binding proteins like DARPins to conveniently add several functional moieties simultaneously for various biomedical applications.



INTRODUCTION

Over the past decade, the highly efficient and selective Cu(I)-dependent azido-alkyne cycloaddition (CuAAC), often denoted by the term "click chemistry", has been introduced to biological molecules.[1] The bio-orthogonal nature of the reaction renders click chemistry ideal for the assembly of bioconjugates in a regio-selective manner, as it allows several simultaneous modifications, avoiding unwanted side products.[2,3]

The covalent conjugation of polyethylene glycol (PEG) to a biomolecule is a clinically validated strategy for the functional modulation of biopharmaceuticals and therefore one of the most relevant covalent modifications of therapeutic proteins.[4,5] For site-specific PEGylation, coupling at Cys is most widely used. Although in principle PEGylation of proteins using CuAAC is straightforward,[6] the biomedical application of these products is limited since an azide or alkyne needs to be introduced into the protein. Furthermore, the instability of Cu(I) makes it challenging to scale up the reaction and maintain high yields. Moreover, CuAAC often requires highly insoluble ligands as chelating additives and Cu(I) is itself potentially cytotoxic and must thus be carefully removed, as it binds to the protein.

Recently, Cu-free click chemistry has been introduced to allow the conjugation of PEG moieties by coupling substituted cyclooctynes and azides without the need for a heavy metal catalyst and additives.[7,8] This strain-promoted azido-alkyne cycloaddition (SPAAC) offers efficient conjugation of azide-labeled proteins under mild conditions. It is thus well-suited for site-specific modification of proteins, particularly of enzymes and binding molecules engineered for therapeutic and diagnostic purposes.[9]

Designed Ankyrin Repeat Proteins (DARPins) have been introduced recently as a class of designed alternative scaffold proteins with features superior over monoclonal antibodies and recombinant antibody fragments.[10] Besides showing high expression yield in *E. coli*, DARPins are very stable, have favorable biophysical properties and carry no cysteine and no essential methionine.[11] Moreover, DARPins of very high affinity can be generated using well-established selection techniques, such as ribosome display and phage display, as previously shown for many different examples.[10] We previously described, among other applications, the successful generation and preclinical testing of DARPins selected for high stability, affinity, and

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tumor targeting directed against the human epidermal growth factor receptor (EGFR),[12] against Her2[13,14] and the Epithelial Cell Adhesion Molecule (EpCAM).[15,16]

DARPins contain no conserved methionine in the randomized repeats, and the only Met present in the original design, besides the initiator codon, is in the N-capping repeat (pos. 34), and can be easily exchanged to leucine. This substitution is suggested by the fact that all following repeats in DARPins have a leucine at the corresponding position. Thus, the resulting protein retains its high solubility, stability, and binding affinity (see below) after the mutation M34L. Some DARPins, such as, e.g., the EpCAM-specific binder Ec1,[16] had already mutated Met34 through the random mutagenesis in ribosome display. Additionally, DARPins are usually expressed with an N-terminal $MRGSH_6$ tag, from which the methionine is not cleaved,[17] and this is also true when an amino acid analogue is introduced.[18] Conversely, if the second amino acid is small, the amino acid analogue will be cleaved off, allowing the introduction of a unique Aha or Hpg at a position elsewhere in the chain.

The click chemistry can be carried out in either orientation, since both the non-natural Met analogues azidohomoalanine (Aha) and homopropargylglycine (Hpg) can be efficiently introduced, simply by exchanging the growth medium shortly before induction and by using a methionine-auxotrophic *E. coli* strain. The resulting "clickable" DARPins can be expressed in high amounts and are capable of bio-orthogonal coupling to "clickable" substrates containing the corresponding azides or alkynes for Cu(I)-dependent coupling or substituted cyclooctynes for Cu(I)-independent click chemistry, respectively.

We demonstrate the utility of this bio-orthogonal coupling by simultaneously derivatizing DARPins with PEG and with a fluorescent dye. Since DARPins are devoid of cysteines, we can introduce a single C-terminal cysteine into Aha-modified clickable DARPins for maleimide coupling of a second tag, in this case a fluorophore. PEGylation has been widely used to extend the serum persistence of proteins.[4,5,13] However, PEGylation can also lead to a decrease in the functional affinity of the molecule to which it is applied, even when the PEG attachment site is most distal to the antigen binding regions.[19] To extend our previous studies, where this phenomenon has been investigated with *in vitro* binding kinetics and mathematical modeling, we now used association and dissociation kinetics determined directly on cells[13,20] by FACS analyses and thus requiring an additional fluorescent label, which must be attached identically in the PEGylated and non-PEGylated molecule.

Here, we present a clickable DARPin recognizing the tumor-associated antigen EpCAM and describe its convenient mono-PEGylation using Cu-free click chemistry at the N-terminus in combination with C-terminal tagging with a fluorescent dye using maleimide coupling. We exploit these doubly modified molecules by showing that PEG has an effect on the on-rate of DARPin binding to cells, but not its off-rate, and also decreases the maximal number of targeting proteins that can be bound to the cell, compared to the non-PEGylated control. This convenient site-specific double tagging may further expand the engineering space of DARPins for biomedical applications.

EXPERIMENTAL PROCEDURES

Materials. If not otherwise stated, all chemicals were obtained from Sigma-Aldrich (Buchs, Switzerland). *E. coli* strain B834 (DE3) (F^- *ompT gal* $hsdS_B$ (r_B^- m_B^-) *met dcm lon* (*lacI, lacUV5-T7 gene 1, ind1, sam7, nin5*)) was from EMD Chemicals, Inc. (U.S.A.) and *E. coli* strain XL1 blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ proAB $lacI^qZ\Delta M15$ Tn10 (Tet^r)]) was purchased from Stratagene (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.). *N*-(3-Aminopropionyl)-5,6-dihydro-11,12-didehydrodibenzo-[b,f]azocine (amino-*aza*-dibenzo-cyclooctyne, DBCO-NH_2) and DBCO-$PEG_{20\ kDa}$ were a kind gift of Click Chemistry Tools (Scottsdale, Arizona, U.S.A.).

Site-Directed Mutagenesis of DARPins Containing Internal Methionine Codons. A single point mutation was introduced into the nonbinding control DARPin N3C[21] to generate the mutation M34L, leading to a gene product devoid of internal methionines. The plasmid pQE30ss-N3C was amplified using the primers 5′-ATCCTGCTGGCTAACGGTGCTGACGTT-3′ and 5′-TAGCCAGCAGGATACGAACTT CGTCGT-3′ followed by a *Dpn*I digest for 2 h. The mutated vector was transformed into chemocompetent *E. coli* XL1 blue cells and single colonies were used to inoculate overnight cultures. After sequencing, a positive clone was subcloned into the expression vector pQIq (a $lacI^q$ encoding variant of pQE30 (Qiagen, Hilden, Germany) with a double stop codon) using *Bam*HI and *Hin*dIII.

Tumor Cell Culture. The EpCAM-positive MCF7 breast carcinoma cell line was obtained from ATCC (American Type Culture Collection, LGC Standards S.à.r.l., Molsheim Cedex, France) and grown in a mixture of Dulbecco modified Eagle's medium (DMEM) (50% v/v) and Ham F12 medium (50% v/v) (Sigma, Buchs, Switzerland) supplemented with 10% fetal calf serum (Amimed, Bioconcept, Allschwil, Switzerland), 100 units/mL of penicillin and 100 μg/mL of streptomycin (Sigma). The cells were incubated at 37 °C in a humidified atmosphere (containing 5% CO_2) and were tested mycoplasma-negative.

Expression and Purification of Clickable DARPins. *E. coli* B834 (DE3) was freshly transformed with the plasmid pQIq encoding anti-EpCAM DARPin Ec1[16] or the off-target control DARPin N3C. The latter is a full-consensus DARPin[21] without any specificity for EpCAM or any other cell-binding activity. A single colony was used to inoculate an overnight culture of 2 × YT medium supplemented with 100 μg/mL ampicillin and 1% glucose. On the next day, 1 L of 2 × YT medium supplemented with 50 μg/mL ampicillin and 1% glucose was inoculated at an optical density at 600 nm (OD_{600}) of 0.1 and grown in a shake flask at 37 °C until an OD_{600} of 1.0−1.2 was reached. The culture was subsequently centrifuged (5000 × *g*, 15 min, 4 °C) and washed thoroughly by resuspending the pellet in ice−cold 0.9% NaCl solution. The washing step was repeated 2 times on ice and the residual pellet transferred into M9 minimal medium (SelenoMethionine Medium Base (Molecular Dimensions LTD, Newmarket, United Kingdom) supplemented with a nutrient mix containing 19 amino acids (Molecular Dimensions Ltd., New Market, Suffolk, U.K.), 50 mg/L ampicillin and 40 mg/L L-azidohomoalanine (Aha) (Bapeks, Riga, Latvia) or L-homopropargylglycine (Hpg) (Chiralix B.V., Nijmegen, Netherlands). The cultures were shaken for 15 min at 30 °C and then induced with 1 mM IPTG. After expression for 4 h, cultures were pelleted by centrifugation (20 min, 5000 × *g*, 4 °C), the pellet was washed with 0.9% NaCl and stored overnight at −80 °C.

After thawing, the bacterial cultures were lysed 3−4 times using a French press (Aminco) at 1200 psi and the DARPin was purified by IMAC.[11] The protein was eluted with PBS_E (1 × PBS, 300 mM imidazole, pH 7.4), dialyzed overnight against 1 × PBS pH 7.4, analyzed by 15% SDS PAGE, and stored at 4 °C.

N-Terminal Sequencing. Incorporation of the non-natural amino acid Aha was verified using N-terminal protein sequencing. A volume of 2 μL DARPin (ca. 5 μg) was diluted

in 100 μL 0.1% TFA and loaded on a Prosorb Sample Preparation Cartridge (ABI). The membrane was washed twice with 0.1% TFA. Edman degradation was performed using a PROCISE cLC492 system to determine N-terminal amino acid residues.

Amino Acid Hydrolysis. The amino acid composition of clickable DARPin was quantified by amino acid hydrolysis using the AccQ Tag Ultra kit (Waters GmbH, Eschborn, Germany). The protein (22.6 μg in 10 μL PBS) was dried followed by a vapor phase hydrolysis in 6 M HCl for 24 h at 110 °C under argon. The dried sample was dissolved in 50 μL borate buffer before 10 μL of the sample were derivatized according to the manufacturer's recommendations. A UPLC system (Waters) was used to separate the amino acids, including norvaline as an internal standard and transferrin as a reference protein.

ELISA. The binding of Aha-Ec1 and Met-Ec1 was compared using an ELISA as previously described.[16]

Synthesis of DBCO-PEG$_{20\ kDa}$. Five milligrams (1 equiv, 13.45 mM) DBCO-NH$_2$ (Click Chemistry Tools, Macon, GA, U.S.A.) was dissolved in 1 mL dichloromethane and 400 mg (1.5 equiv, 20 mM) NHS-mPEG$_{20\ kDa}$ (JenKem Technology Inc., U.S.A.) was added in 3 mL dichloromethane. This solvent was used to decrease viscosity. The reaction was stirred overnight at room temperature under N_2 atmosphere, and an aliquot was analyzed using thin layer chromatography (TLC) and ninhydrin staining. Finally, the reaction was quenched with a 1000-fold molar excess of ethylene diamine (4 h, at RT), followed by evaporation of the organic solvent under vacuum (Rotavapor, Büchi, Flawil, Switzerland). The resulting modified PEG polymer was redissolved in ddH_2O and lyophilized to complete dryness for 48 h in a freeze−dryer (Brouwer, Rotkreuz, Switzerland). During the course of this study, purified DBCO-PEG$_{20\ kDa}$ became commercially available (Click Chemistry Tools) and was also used for PEGylation of clickable DARPins.

Cu-Free Click PEGylation of DARPins. The clickable DARPin Aha-Ec1 was reacted with a 2-fold molar excess of DBCO-PEG$_{20\ kDa}$ at 4 °C and aliquots were taken over time, snap frozen, and stored at −20 °C. The time-dependent reaction was analyzed by 15% SDS PAGE and Coomassie staining.

Dual-Tagging of DARPins by Conjugation with Alexa488 and DBCO-PEG$_{20\ kDa}$. For dual-tagging, the clickable DARPin Aha-Ec1 containing a C-terminal cysteine was expressed, purified, dialyzed, and stored frozen as described above. The DARPin (12 mg/mL in PBS) was thawed on ice and reduced for 30 min at 37 °C with 50 mM Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP, Pierce, Thermo Fisher Scientific, Lausanne, Switzerland) followed by desalting on a PD-10 column (GE Healthcare Europe GmbH, Switzerland), equilibrated with freshly degassed and N_2-flushed PBS pH 7.2. The concentrations of the desalted DARPins were determined at 280 nM (Nanodrop 1000, Thermo Scientific, Switzerland) (usually around 300 μM) and a 2-fold molar excess of Alexa Fluor 488 C5 maleimide (Invitrogen, Life Technologies Europe BV, Zug, Switzerland) was added in typically 100 μL DMF. The dye was coupled for 1.5 h at 37 °C and quenched with a 10-fold molar excess of DTT. The conjugate was further purified by anion exchange chromatography with a MonoQ GL 5/50 column connected to an Äkta Explorer FPLC device (GE Healthcare Europe GmbH, Glattbrugg, Switzerland). The protein was diluted in buffer A (50 mM Hepes, 20 mM NaCl, pH 8.5), loaded on the MonoQ column and eluted with a gradient of buffer B (50 mM Hepes, 1 M NaCl, pH 8.5). The fractions containing labeled DARPin were pooled and concentrated (Amicon Ultra-4 Centrifugal Filter Unit, MWCO 3 kDa, Millipore, U.S.A.) according to the manufacturer's protocol.

An aliquot of the Alexa-labeled DARPin (typically around 300 μM) was mixed with a 2-fold molar excess of DBCO-PEG$_{20\ kDa}$ (Click Chemistry Tools) from a 5 mM stock in PBS and vortexed. The click reaction was performed at 4 °C for 72 h in PBS pH 7.2 without agitation, and PEGylated DARPin was separated from free DBCO-PEG$_{20\ kDa}$ and non-PEGylated DARPin using anion exchange chromatography (MonoQ GL 5/50) sequentially equilibrated with buffers A and B via a step gradient. The PEG$_{20\ kDa}$-DARPin-Alexa488 conjugate was further concentrated using an Amicon centrifugal filter unit (MWCO 3 kDa, Millipore, U.S.A.) before the quality was assessed by 15% SDS PAGE in combination with a fluorimeter (LAS 3000, Fujifilm Europe GmbH, Düsseldorf, Germany) followed by Coomassie staining. The protein concentration was quantified using the Pierce 660 nm protein assay kit (Pierce) and unlabeled Ec1 to generate a standard curve as reference. Dual tagged DARPins were stored in the dark at −80 °C until use.

Analytical Gel Filtration. Unconjugated and PEG-conjugated DARPins were evaluated by analytical gel filtration with a Superdex 200 PC3.2/30 column in combination with an Äkta Micro FPLC (GE Healthcare Europe GmbH, Switzerland). Degassed and sterile filtered PBS pH 7.2 was used as running buffer. A standard containing β-amylase, BSA, and cytochrome *c* was used to determine the apparent molecular weights ($M_{w,app}$) of the DARPin.

Preparation of Cells for Affinity Measurement. Affinity measurements with DARPins on cells were performed using a Partec CyFlow Space flow cytometer (Partec GmbH, Görlitz, Germany). MCF7 cells were grown as described above to a confluency of 70%. The cells were washed twice with PBS and detached with trypsin-EDTA, followed by resuspension in PBS-BA (1 × PBS, 1% BSA, 0.2% NaN_3). Cells were quantified with a CASY cell counter (Roche Diagnostics GmbH, Germany) and incubated for 30 min at 37 °C in this azide-containing buffer to inhibit further receptor internalization.

Determination of Dissociation Rates from Cells. After centrifugation (300 × *g*, 3 min, RT), 0.75 × 10^6 cells/mL were resuspended in PBS-BA containing 100 nM Ec1-Alexa488, PEG$_{20\ kDa}$-Ec1-Alexa488, or the nonbinding controls N3C-Alexa488 or PEG$_{20\ kDa}$-N3C-Alexa488, which were used to assess nonspecific background binding on cells. Cells were incubated for 1.5 h at 4 °C on a rocker to allow complete saturation with the binder. A typical off-rate experiment was started by centrifugation of cells (300 × *g*, 3 min, RT) followed by removal of the supernatant and resuspension of the pellet in PBS-BA supplemented with 1 μM unlabeled Ec1 as competitor to prevent rebinding. Untreated cells were used to determine background autofluorescence. The median fluorescence intensities (MFI) of the time points (corrected for any sample preparation time) were determined using the *FlowJo* software (v 7.6.4, Treestar Inc., U.S.A.). The data was plotted using *Prism* (v 5.04, GraphPad Software Inc., U.S.A.) and all curves were fitted using a monoexponential decay function.

Determination of Association Rates on Cells. A final concentration of 67.5 nM, 22.5 nM, 7.5 nM, and 2.5 nM of Ec1-Alexa488 and PEG20$_{kDa}$-Ec1-Alexa488 was prepared in 6 mL PBS-BA at room temperature. The association measurement was started by addition of 2.1 × 10^6 MCF7 cells in 1 mL PBS-BA (final concentration 3 × 10^5 cells/mL, total volume 7 mL for each construct and concentration). N3C-Alexa488 and PEG$_{20\ kDa}$-N3C-Alexa488 were used as controls to assess

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nonspecific background binding on cells. The MFI of the data points were determined as described above and fitted to a mono-exponential function of time using the *Prism* software. The corresponding k_{off} measured in the dissociation experiment on cells for each binder was used as a constraint to calculate k_{on}.

RESULTS

Expression and Purification of Clickable DARPin Ec1c. To obtain a unique label at the N-terminus of the DARPin, we introduced the non-natural amino acid azidohomoalanine (Aha), a methionine mimic, to allow modification by bio-orthogonal click chemistry. We used the methionine-auxotroph *E. coli* strain B834 (DE3) and exchanged the medium to M9 minimal medium supplemented with Aha to allow quantitative incorporation of Aha at amino acid position one. Since the second amino acid is Arg, the initiator Met is not cleaved.[17,18] Freshly transformed cells were first grown in 2 × YT medium to obtain fast growth rates and high cell densities, followed by washing to deplete extracellular methionine from the expression cultures. After inoculation into M9 minimal medium, residual intracellular methionine pools were additionally decreased by a delayed induction of the cultures with IPTG. Expression of DARPin was shown to continue in a time-dependent manner as monitored by SDS PAGE (SI Figure S1). After IMAC purification, a yield of 30 mg of protein per liter culture in shake flasks was determined (SI Figure S2).

Analysis of Clickable DARPin. The IMAC-purified clickable DARPin was analyzed by N-terminal Edman sequencing in comparison to unmodified DARPin Ec1 (SI Figure S3). In the latter, methionine gave a specific peak with a retention time of 17.5 min, whereas this peak was decreased to background levels in the chromatogram of clickable DARPin. For these proteins, Edman degradation revealed a new peak for the first N-terminal amino acid with a retention time of approximately 16 min, which can be assigned to the non-natural amino acid Aha. The N-terminal sequence was Aha-Arg-Gly-Ser, with a minor fraction starting with the sequence Arg-Gly-Ser, indicating that the N-terminal Aha was cleaved off. The proteins were completely devoid of methionine. Conventionally expressed DARPin showed the amino acid sequence Met-Arg-Gly-Ser (with a minor fraction starting with Arg-Gly-Ser) without the appearance of a peak at 16 min, which was seen with the protein prepared from *E. coli* grown in the presence of Aha.

The single introduction of Aha into the DARPin and the absence of methionines was further confirmed by amino acid analysis after total hydrolysis (SI Table S1). A complete absence of methionine in the Aha-containing DARPins was determined, whereas one methionine, the initiator codon, was present in conventionally expressed DARPin Ec1, as expected from the amino acid sequence. Both Aha- and Met-containing DARPins were further compared by ELISA. Binding to EpCAM was fully retained after the introduction of the non-natural amino acid at position 1 (SI Figure S4).

In addition, we expressed Hpg-containing DARPins using the medium exchange method. Both Hpg-DARPins and Aha-DARPins were reactive under CuAAC conditions using Cu(I) as catalyst, and could thus be coupled to each other, forming a covalent dimer (SI Figure S5), while suitable controls showed no dimer formation.

Synthesis of DBCO-PEG$_{20\ kDa}$. DBCO-PEG$_{20\ kDa}$ was prepared from DBCO-NH$_2$ and NHS-mPEG. The resulting polymer was devoid of free amino groups, as analyzed by TLC and ninhydrin staining, demonstrating that DBCO-NH$_2$ had quantitatively reacted with the excess of NHS-mPEG overnight (Figure 1). After quenching with ethylene diamine and lyophilization, the generated DBCO-PEG$_{20\ kDa}$ was used for Cu-free PEGylation of "clickable" DARPin.

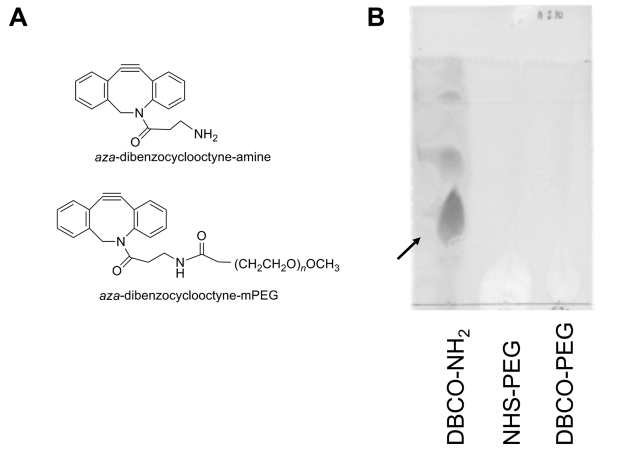

Figure 1. (A) Chemical structure of DBCO-NH$_2$ and DBCO-PEG. (B) Analysis of DBCO-PEG using thin layer chromatography and ninhydrin staining.

Bio-Orthogonal Coupling of Clickable DARPins Using Cu-Free Click Chemistry. The DBCO-PEG$_{20\ kDa}$ was coupled to "clickable" DARPins resulting in PEGylation exclusively at the N-terminus. A 2-fold molar excess of DBCO-PEG$_{20\ kDa}$ was found to be a good compromise to maximize reactivity and minimize viscosity of the reaction mixture, and was thus used for further reactions (SI Figure S6). "Clickable" DARPins reacted with DBCO-PEG$_{20\ kDa}$ in several buffers and pH values tested almost to completion over 48 h at 4 °C (data not shown) without agitation. PEGylation with DBCO-PEG$_{20\ kDa}$ led to a distinct mono-PEGylated product, monitored as a shift to a single band by SDS PAGE in a time-dependent manner (Figure 2).

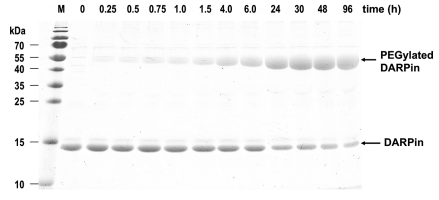

Figure 2. PEGylation of 100 μM Aha-Ec1 using a 2-fold molar excess of DBCO-PEG$_{20\ kDa}$ as a function of time at 4 °C. The conversion to PEGylated DARPin was analyzed by 15% SDS PAGE.

Preparation of Ec1-Alexa488 and PEG$_{20\ kDa}$-Ec1-Alexa488 for Affinity Measurements. N-terminally "clickable" DARPins, which also carried a single C-terminal cysteine-tag (Gly-Gly-Cys), were expressed using the medium exchange method described above for incorporation of Aha to allow site-specific PEGylation via click chemistry, and Alexa488-labeling on the same molecule via thiol chemistry (Figure 3). First, the IMAC-purified clickable DARPin was completely reduced, since disulfide bridged dimers form during the production. To prevent

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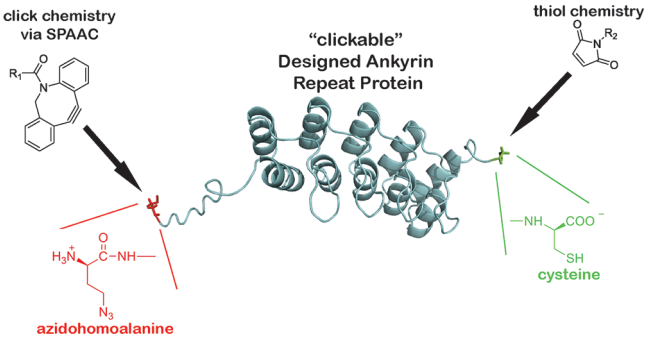

Figure 3. Schematic illustration of the EpCAM-targeting "clickable" DARPin Ec1. The protein can be labeled N-terminally via click chemistry and C-terminally via thiol chemistry using different conjugates.

interference of a high excess of TCEP with the coupling reaction, the samples were desalted prior to the addition of Alexa dye in degassed PBS. Ec1-Alexa488 was subsequently purified using anion exchange chromatography to separate the product from free dye and unlabeled DARPin. As shown in SI Figure S7A, the labeled protein eluted at higher salt concentration than the unlabeled one as a consequence of the increased negative charge of Ec1-Alexa488.

In a second step, one-half of the resulting Ec1-Alexa488 was N-terminally PEGylated using DBCO-$PEG_{20\,kDa}$, leading to a mono-PEGylated product. The Cu-free click-PEGylation was run for 72 h at 4 °C allowing high conversion of the DARPin to the PEGylated form. The reaction mixture was purified by anion exchange chromatography (SI Figure S7B) as described elsewhere,[22] concentrated and analyzed by 15% SDS PAGE. All constructs were obtained at high purity with the expected band for the mono-PEGylated DARPins Ec1 and N3C (Figure 4).

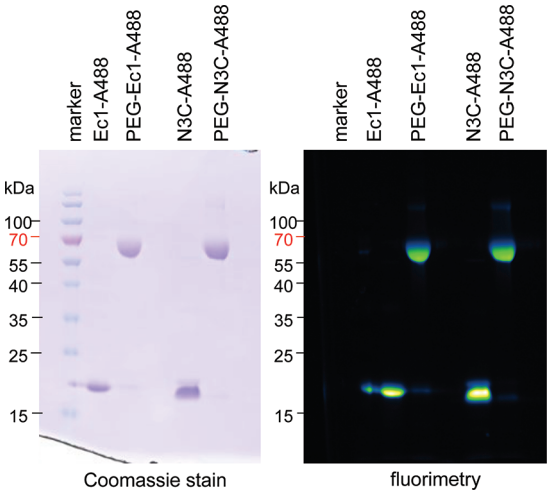

Figure 4. SDS PAGE analysis of non-PEGylated and PEGylated Alexa488-labeled DARPins Ec1 and N3C used for affinity measurements on cells. The proteins were analyzed by Coomassie stain (left) and fluorimetry to detect Alexa488 (right).

Analytical Gel Filtration. Ec1-Alexa488 and $PEG_{20\,kDa}$-Ec1-Alexa488 were analyzed by gel filtration in order to determine the increase in hydrodynamic radius caused by mono-PEGylation at the N-terminus (SI Figure S8). Non-PEGylated Ec1-Alexa488 eluted with a retention volume of 1.81 mL ($M_{w,app}$ = 17.4 kDa). PEGylation of Ec1-Alexa488 lead to an increase in its hydrodynamic radius with a retention volume of 1.31 mL ($M_{w,app}$ = 250 kDa). It is well-known[23] that the hydrodynamic radius of a PEGylated protein is much larger than its nominal M_w of 19 + 20 = 39 kDa.

Binding Kinetics: Determination of the Dissociation and Association Rates on Cells. $PEG_{20\,kDa}$-Ec1-Alexa488 and Ec1-Alexa488 were used as fluorescent probes to determine the dissociation rates from MCF7 breast carcinoma cells. For both binders, nearly identical k_{off} values were determined (Ec1-Alexa488: 2.35 × 10^{-5} s^{-1}; $PEG_{20\,kDa}$-Ec1-Alexa488: 1.96 × 10^{-5} s^{-1}). However, the maximum MFI differed for both constructs, with PEGylated DARPins reaching a maximal intensity of only 65% of the original signal of the non-PEGylated DARPins (Figure 5).

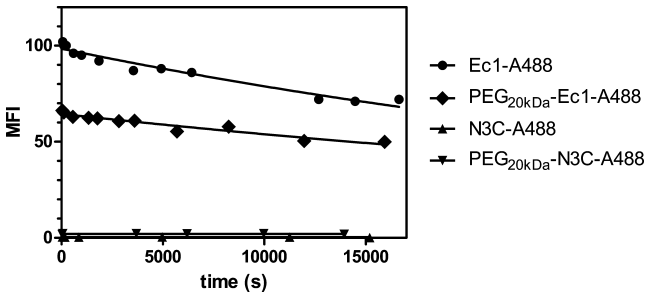

Figure 5. Determination of dissociation rates of PEGylated and non-PEGylated Ec1 from MCF7 cells.

The association rates of PEGylated and non-PEGylated DARPins were also assessed by flow cytometry and monitored over time using four different concentrations of DARPins (Figure 6). Non-PEGylated Ec1-Alexa488 associated with a k_{on}

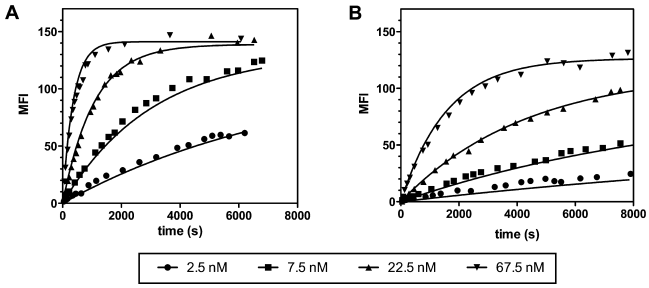

Figure 6. Determination of the association rate of Ec1-Alexa488 (A) and $PEG_{20\,kDa}$-Ec1-Alexa488 (B) on MCF7 cells.

of 4.0 × 10^4 M^{-1} s^{-1}. In contrast, conjugation of a single $PEG_{20\,kDa}$ polymer at the N-terminus led to a decrease in association rates with a k_{on} of 8.9 × 10^3 M^{-1} s^{-1}. The off-target control DARPins N3C-Alexa488 and $PEG_{20\,kDa}$-N3C-Alexa488 showed only minor background binding on MCF7 cells.

The overall K_D of Ec1-Alexa488 on the tumor cells was thus determined from k_{off}/k_{on} as 578 pM. Mono-PEGylation of Ec1-Alexa488 resulted in a decreased affinity with a K_D of 2.18 nM (Table 1).

DISCUSSION

There is growing interest in using alternative scaffolds as binding proteins for biomedical applications as they show distinct advantages over monoclonal antibodies and recombinant antibody fragments, particularly with respect to options for rational engineering, provided the proteins are very robust.[24] As an

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Table 1. Results from Affinity Measurements on MCF7 Cells Using Flow Cytometry

	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (M)	fold change (over Ec1)
Ec1-Alexa488	4×10^4	2.3×10^{-5}	5.8×10^{-10}	-
$PEG_{20\ kDa}$-Ec1-Alexa488	8.9×10^3	2.0×10^{-5}	2.2×10^{-9}	3.8

example, DARPins can be conveniently engineered to well expressing, stable high-affinity binders in mono- and multispecific formats and fusion proteins, and they can be used for tumor targeting either as pure binding proteins to inhibit growth factor receptor signaling, or as bioconjugates equipped with various effector functions.[10]

Performance and Analysis of the Click Chemistry with DARPins. Site-directed coupling of two or more different moieties to one and the same binding protein would increase its modularity and functional spectrum for biomedical applications. For example, a DARPin might be conjugated to a drug to deliver antitumor activity, and it could be further equipped with a PEG moiety at a distinct site to modify its pharmacokinetic properties.[4] DARPins have no natural cysteines, and thus single Cys residues can be easily engineered into suitable positions to enable site-specific conjugation with various effector moieties, typically by using maleimide chemistry, to couple chelators for radiolabeling, liposomes, PEG, or cytotoxic anticancer agents.[25]

However, the problem comes with introducing the second conjugate. If two cysteines were used, difficult separations of positional isomers need to be established. If lysines are used, heterogeneity is almost unavoidable.[26] If the N-terminal amino group is used, which is privileged because of greater reactivity than lysines,[6,20,27] reaction conditions and separation must be optimized to avoid reaction with lysines as a side reaction and/or large amounts of nonreacted protein.

Here, we report that orthogonal click chemistry together with site-specific maleimide linkage is fully compatible with engineering different effector functions into one binding protein to obtain a heterobifunctional bioconjugate. We demonstrate this with an EpCAM-specific DARPin (Ec1) as an example of a binding protein selected for tumor targeting.[16] First, maleimide-Alexa488 was coupled to a C-terminal cysteine and purified using anion exchange chromatography, before in a second step the fluorescent DARPin was PEGylated with the polymer DBCO-$PEG_{20\ kDa}$ using Cu-free click chemistry. The so-modified Ec1 DARPin was fully functional and used in this study to determine the effect of PEGylation on binding to EpCAM on tumor cells without the shortcomings of conventional unspecific labeling techniques.[1,26]

The copper(I)-catalyzed azide−alkyne [3 + 2] cycloaddition (CuAAC) reaction was described as a pioneering platform for the synthesis of orthogonal conjugates and could prove useful for site-specific modification of therapeutic proteins as shown for superoxide dismutase[6] or antibody fragments.[28] However, even in well purified preparations, contamination by potentially cytotoxic copper, reducing agents, and poorly soluble Cu(I)-stabilizing chelating ligands, required to catalyze the triazole formation, limits biomedical applications and may interfere with the protein's activity.[29] Recently, reagents for strain-promoted azido-alkyne cycloaddition (SPAAC) became available that have enabled site-specific coupling under mild conditions without the shortcomings of CuAAC mentioned above.[30] The click reactions between substituted cyclooctynes and azides have now repeatedly been described as a good alternative for efficient and specific protein labeling *in vitro*[7] and *in vivo*.[8,31] Furthermore, numerous linker constructs have become available promoting fast conversion reactions in aqueous solution. One of these compounds, *aza*-dibenzocyclooctyne (abbreviated either as DIBAC or DBCO), offers favorable properties for click conjugation. Recently, a DBCO-$mPEG_{2\ kDa}$ moiety was generated for PEGylation of Aha-CalB, an azido-containing lipase, using Cu(I)-free click chemistry without loss of enzymatic activity. Unfortunately, the study unveiled increased stoichiometries of PEG attached to the protein, which occurred in a time-dependent manner. In this case, the presence of five ATG codons in the CalB gene resulted in the metabolic introduction of five Aha's into the enzyme during expression, responsible for the limited control over PEGylation stoichiometries and attachment sites.[9,32] Thus, a protein with multiple methionine codons would not provide an ideal basis to selective site-specific labeling.

DARPins have no methionine in the internal repeats, and the one in the original N-cap design (M34) can be replaced by a leucine—a residue found at the corresponding position in the internal repeats—to give a fully equivalent molecule. In general, the occurrence of methionines in the randomized positions of DARPins is very low, an excellent prerequisite for the introduction of clickable methionine surrogates at well-defined positions and quantities for practically all members of the library. The choice of the second amino acid after the initiator codon determines whether the first amino acid is cleaved off or not.[17,18] If Aha is to be introduced at another position, a small amino acid has to be chosen for position 2.

With other proteins, such as antibody fragments, this N-terminal labeling strategy is more challenging due to their high content of internal methionines[28] and the additional complication with the cleavable signal sequence needed for transport to the periplasm, or, in its absence, the necessity of refolding.

Here, we expressed "clickable" DARPins using a medium exchange method for the introduction of Aha specifically at the N-terminus. Despite the use of minimal medium, up to 30 mg/L soluble protein was obtained from *E. coli* cultures after IMAC purification. Since DARPins in general can be expressed in high amounts, this property inherent to DARPins seems to be maintained even in the presence of the unnatural amino acid.[11,16] The "clickable" DARPins did not show any difference from conventionally expressed DARPins as analyzed on SDS PAGE, or on gel filtration or ELISA (SI Figures S2, S4, S8). Approximately 85% of the DARPin reacted with PEG, and we assume that the remaining 15% had lost the N-terminal Aha due to processing of the primary amino acid, presumably by *E. coli* methionine amino-peptidase,[18] as detected by N-terminal sequencing. The click reaction itself is thus essentially complete.

In summary, the click chemistry leads to very clean products and convenient regiospecific labeling, and in conjunction with maleimide−thiol chemistry, to a facile and versatile double labeling procedure suitable for scale-up.

Analysis of Binding Kinetics of PEGylated DARPins on Cells with Doubly Labeled DARPins. Being able to introduce PEG at the N-terminus of a DARPin at a single functional azide together with Alexa488 at a C-terminal cysteine permitted us to measure association and dissociation kinetics on tumor cells. PEGylated and non-PEGylated DARPins showed similar dissociation rates, which we previously also reported for PEGylated Her2-binding antibody fragments using surface plasmon resonance measurements.[19] On the other hand, comparison of the absolute levels of bound PEGylated and non-PEGylated

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DARPins unveiled a difference, suggesting that the number of receptors accessible to the PEGylated DARPin was reduced due to steric hindrance by the PEG tails of already surface-bound DARPins, resulting in dynamic blocking effects.[19] Once the DARPin binds to its target, the conjugated PEG probably shields neighboring epitopes on the cells, resulting in *inter*molecular inhibition, without affecting the dissociation rate constant k_{off}.

Determination of the association rate constant k_{on} of PEGylated DARPins on cells by using FACS identified a clear difference between non-PEGylated and mono-PEGylated binders, with the PEGylated DARPins associating with a 3.8-fold slower rate, probably due to the *intra*molecular shielding effects by the large hydration shell provided by the PEG moiety.[5] Since the off-rate is unchanged and the on-rate is reduced, the resulting affinity of the PEGylated DARPin Ec1 on EpCAM-positive cells was reduced 3.8-fold, compared to the non-PEGylated counterpart (2.18 nM vs 578 pM, respectively).

Previously, studies of the effect of PEG on the on-rate and off-rate of binding proteins were limited to label-free methods such as surface plasmon resonance.[19] However, being now able to produce binding molecules that can simultaneously be conjugated via thiol and click chemistries allowed us to study these and other phenomena directly on cells in detail.

Our finding on cells are fully consistent with those previously found *in vitro*.[19] Without the double labeling, only the equilibrium binding on cells could previously be measured, showing a 5-fold reduced binding affinity after PEGylation, which is again fully consistent with our current findings. The fact that these effects are observed for DARPins and for antibody scFv fragments also emphasizes that they are generic and determined by the properties of PEG, and much less by the properties of the protein. Briefly, in all these cases the PEG was attached at a position most distal from the target binding site and PEG did not statically prevent binding in any way. Also, the population of molecules was fully functional, i.e., all binding molecules (DARPins and scFv) could be bound to the target when target was in excess.[19] Furthermore, the binding site was not distorted, since the off-rate was identical within experimental error.

Our data are consistent with the previous notion that PEGylation has two intrinsic effects: First, it transiently and dynamically blocks the binding site, decreasing the number of binders which can associate at any one time, thereby decreasing the observed on-rate. This is not an effect of diffusion, as the off-rate is not influenced, as detailed previously.[19] Since ultimately all molecules can bind, this inhibition is transient, and is only seen in the association kinetics. We have termed this *"intra*molecular blocking".

The second effect comes into play after binding to a surface, previously the surface on a BIACORE chip,[19] in the present work a cell surface. The PEG moiety prevents binding to epitopes on neighboring molecules, thereby decreasing the total amount of available binding epitopes. This effect also influences the observed kinetics at intermediate times.[19] We have termed this *"inter*molecular blocking".

We have thus shown for the first time directly that these effects are also operational on cells. We hypothesize that, by the nature of these effects, this will be observed for any PEGylated protein, and thus a small decrease of on-rate—and consequently of affinity—is intrinsic to the derivatization with a hydrophilic tail, while the effect on the saturation level of binding will depend on the receptor density of the cell.

CONCLUSIONS

In summary, the compatibility of the DARPin format with click chemistry and site-specific maleimide coupling together with an expanding amount of commercially available moieties for bioconjugation holds promise for tailor-made functionalization of these binding proteins. Potential fields of biomedical application include tumor targeting with therapeutic and diagnostic payloads, such as "click-ready" nanoparticles,[33] virus-like particles,[34] dendrimers,[35] radioisotopes,[36] or fluorophores,[37] but also applications in making diagnostic devices, where specifically derivatized DARPins may have to be coupled in a site-directed manner to a surface.

ASSOCIATED CONTENT

Supporting Information

One supplementary table, and 8 supplementary figures document purification and analysis of the bioconjugates. This material is available free of charge via the Internet at http://pubs.acs.org.

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