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Approach for Half-Life Extension of Small Antibody Fragments That Does Not Affect Tissue Uptake

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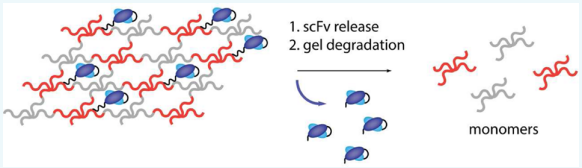
Supporting Information

ABSTRACT: The utility of antigen-binding antibody fragments is often limited by their short half-lives. Half-life extension of such fragments is usually accomplished by attachment or binding to high-molecular-weight carriers that reduce the renal elimination rate. However, the higher hydrodynamic radius results in greater confinement in the vascular compartment and, thus, lower tissue distribution. We have developed a chemically controlled drug delivery system in which the drug is covalently attached to hydrogel microspheres by a self-cleaving β-eliminative linker; upon subcutaneous injection, the $t_{1/2,\beta}$ of the released drug acquires the $t_{1/2}$ of linker cleavage. In the present work, we compared the pharmacokinetics of an anti-TNFα scFv, the same scFv attached to 40 kDa PEG by a stable linker, and the scFv attached to hydrogel microspheres by a self-cleaving linker. We also developed a general approach for the selective attachment of β-eliminative linkers to the N-termini of proteins. In rats, the scFv had a $t_{1/2,\beta}$ of 4 h and a high volume of distribution at steady state ($V_{d,SS}$), suggesting extensive tissue distribution. The PEG−scFv conjugate had an increased $t_{1/2,\beta}$ of about 2 days but showed a reduced $V_{d,SS}$ that was similar to the plasma volume. In contrast, the tissue-penetrable scFv released from the hydrogel system had a $t_{1/2,\beta}$ of about 2 weeks. Thus, the cleavable microsphere−scFv conjugate releases its protein cargo with a prolonged half-life comparable to that of most full-length mAbs and in a form that has the high tissue distribution characteristic of smaller mAb fragments. Other antigen-binding antibody fragments should be amenable to the half-life extension approach described here.



INTRODUCTION

Antigen-specific fragments of monoclonal antibodies (mAbs) are in wide use for a variety of purposes. A major benefit of smaller sized fragments compared to full-sized mAbs is their enhanced penetration into tissues.[1] However, because such fragments lack the FcR-mediated recycling of mAbs and readily undergo renal elimination, they have short circulating half-lives, usually measured in minutes to hours.

Strategies developed to extend the half-lives of small antibody fragments primarily involve permanent covalent conjugation or binding to high-molecular weight carriers such as polyethylene glycol (PEG), albumin, or albumin-binding proteins.[2,3] The increased size conferred to the fragment by the carrier, or to a macromolecule to which it binds, reduces the rate of renal elimination and, hence, increases circulating lifetime. A deficiency of the approach is that the higher hydrodynamic radius of such conjugates may confine the fragment to the vascular compartment and thus negate the benefit of high tissue penetration of a small mAb fragment.

We have developed an approach for half-life extension whereby a drug is covalently tethered to a long-lived macromolecular carrier by a linker that slowly self-cleaves to release the free drug.[4,5] Here, a macromolecular carrier is attached to a linker that is connected to a drug via a carbamate group (**1**; Scheme 1). The α-carbon has an acidic carbon−hydrogen bond (C−H), the pK_a of which is controlled by an electron-withdrawing pK_a "modulator" (Mod). Upon hydroxide ion-catalyzed proton removal to give **2**, a rapid β-elimination occurs to cleave the linker−carbamate bond and release the free drug and a substituted alkene **3**. The rate of drug release is proportional to the acidity of the proton, which is controlled by the chemical nature of the modulator; thus, the release rate of the drug is controlled by the modulator. The linker cleavage is controlled only by pH and is not susceptible to enzyme catalysis.

The carrier may be a circulating macromolecule such as polyethylene glycol for half-life extensions of about 1 week,[4,6,7] an approach that is limited by renal elimination of the conjugate. Alternatively, the carrier can be a subcutaneously injected noncirculating hydrogel (HG) depot, where the $t_{1/2,\beta}$ of the released drug tracks the rate of linker cleavage and permits half-life extensions of weeks to months.[5,8,9] β-Eliminative linkers with a longer cleavage $t_{1/2}$ are incorporated into each of the hydrogel cross-links, so the gels degrade after the drug is released. Because this technology releases the drug originally attached to the carrier, it should be particularly useful for half-life extension of small antibody-fragments without

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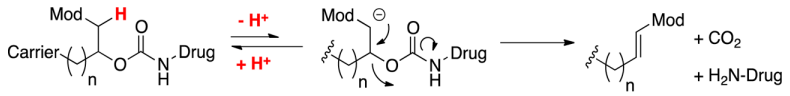
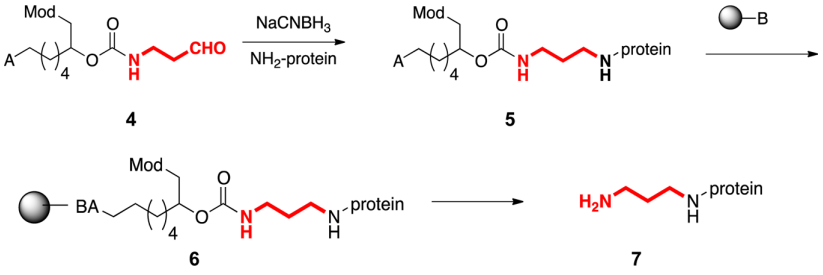
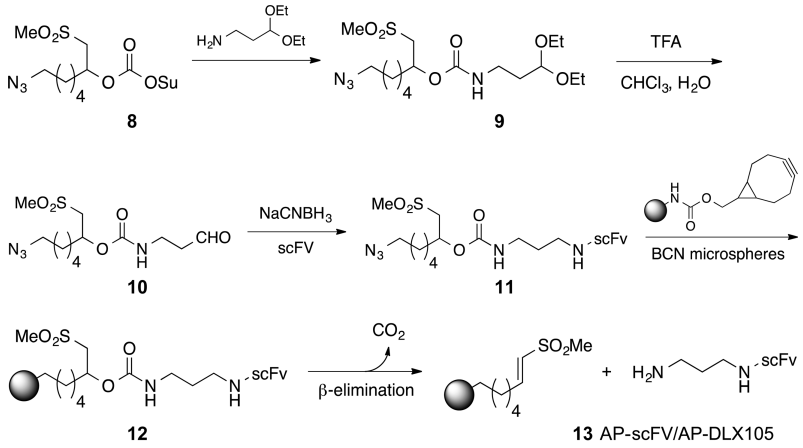
Scheme 1. Modulator-controlled Drug Release from β-eliminative Linkers



Scheme 2. General Strategy for N-terminal Attachment of a Protein to and Release from a Macromolecular Carrier



Scheme 3. Preparation and Subsequent Cleavage of N-terminally Linked DLX105 scFv Microspheres



increasing their size; thus, the benefit of higher tissue penetration of the smaller size fragments should be retained.

Thus far, we have reported the use of β-eliminative linkers for half-life extension of peptides up to ~40 amino acids[4,5,7,9] and a small molecule.[6] In the present work, we applied our linker technology to DLX105,[10] a 246 amino acid 26.3 kDa univalent single-chain variable fragment (scFv) antibody that binds tightly to and neutralizes human tumor necrosis factor alpha (TNFα). We attached DLX105 to two carriers: a circulating 40 kDa PEG via a conventional stable linker and 40 μm tetra-PEG hydrogel microspheres by a self-cleaving β-eliminative linker. Both conjugates relied on bifunctional azido-aldehyde linkers to facilitate selective reductive alkylation of the α-amino group of the protein. In the rat, the stable PEG–scFv conjugate prolonged the half-life of the attached scFv from about 4 h to about 2 days; however, the volume of distribution at steady state ($V_{d,SS}$) was greatly reduced compared to the free scFv, indicating confinement of the large conjugate to the vascular compartment and low tissue distribution. In contrast, the releasable hydrogel microsphere–scFv conjugate conferred a long half-life of about 2 weeks to the released protein, which has high tissue distribution.

RESULTS AND DISCUSSION

Chemistry. A new, general strategy was developed to enable selective attachment of β-eliminative linkers to the N-termini of proteins. As shown in Scheme 2, 3-aminopropionaldehyde is incorporated into the scissile carbamate of a β-eliminative linker **4** that contains an additional connecting group, A, at the opposite terminus. The linker-aldehyde **4** is selectively and stably attached to the N-terminus of a protein by reductive alkylation with $NaCNBH_3$[11] and then connected to a carrier via orthogonal reaction with group B to give conjugate **6**. Upon β-elimination, the scissile carbamate is cleaved, transferring the 3-aminopropyl remnant to the N-terminus of the protein, **7**. Of course, in this approach, the small remnant transferred to the protein must not detrimentally affect its properties. The specific method used for synthesis of intermediates and coupling of the hydrogel microsphere carrier to the α-amino group of DLX105 is illustrated in Scheme 3.

First, the bifunctional azido aldehyde linker, **10**, was prepared by reaction of *O*-(7-azido-1-methylsulfonyl-2-heptyl)-*O*′-succinimidyl carbonate, **8**,[6] with 3-aminopropionaldehyde diethyl acetal, followed by TFA-promoted hydrolysis to give the aldehyde **10**. The $MeSO_2$ modulator was chosen because it has an in vivo cleavage $t_{1/2}$ of about 2 weeks.[4]

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Second, the linker **10** was coupled to the scFv by selective reductive alkylation of the α-amine using $NaCNBH_3$.[12] The reductive alkylation of DLX105 was optimized by varying **10** for optimal yield of the mono- versus multialkylated protein at the expense of unreacted protein. In this manner, mixtures could be directly coupled to a carrier with minimum interference of multialkyl protein, and unreacted protein could be removed by washing the microspheres. Analysis of the stoichiometry of reductive alkylation was accomplished by a PEG-shift sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) assay of azido-linker−scFv. Reductive alkylation reaction mixtures were subjected to strain-promoted alkyne−azide cycloaddition (SPAAC) with DBCO−$PEG_{5\ kDa}$ and assayed for shifts in migration by SDS-PAGE. Attachment of a 5 kDa PEG group to an azido-linker−scFv decreased the SDS-PAGE mobility of the conjugate by ∼15 kDa for each attached 5 kDa PEG. After optimization of reaction conditions, 50 mg of the scFv was treated with 1.5 equiv of aldehyde **10** and 10 mM $NaCNBH_3$ at pH 6.0. PEG-shift SDS-PAGE analysis showed 54% conversion of the scFv giving a 46:42:12 mixture of unmodified−monoalkyl−dialkyl DLX105 (Figure 1), indicating that almost 80% of the alkylated product was the monoalkylated scFv **11**.

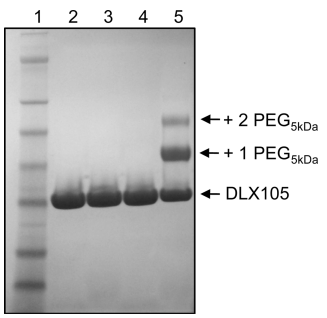

Figure 1. SDS-PAGE of PEG-shift assay of the $NaCNBH_3$ reductive amination of DLX105 with **3**. Lanes: (1) Thermo SeeBlue Plus2 molecular weight markers (from top in kDa: 191, 98, 62, 49, 38, 28, 17, 14, and 6); (2) DLX105; (3) DLX105 treated with DBCO−$PEG_{5\ kDa}$; (4) DLX105 reductive alkylation reaction; and (5) DLX105 reductive alkylation reaction in lane 4 treated with DBCO−$PEG_{5\ kDa}$.

Finally, bicyclononyne (BCN)-derivatized tetra-PEG microspheres[8] were treated with the unpurified N_3-linker−scFv mixture, **11**, for 48 h, at which time the A_{280} in the supernatant was constant; note that only the azide-linker-derivatized scFvs can react with the modified microspheres. The microspheres were then washed to remove unreacted scFv and **11**.

Base-catalyzed β-eliminative cleavage of **12** releases the scFv with a short amino-propyl remnant on the N-terminus (**13**, AP-scFv). The DLX105-microspheres **12** showed complete release of 8 nmole (2.8 mg) of AP-scFv/mL packed slurry with a $t_{1/2}$ of 11 h at pH 9.4, corresponding to a $t_{1/2}$ of ∼1100 h at pH 7.4, 37 °C.[5] Because the in vivo $t_{1/2}$ is generally ∼3-fold lower than the in vitro value,[4] we anticipated this linker would show an in vivo $t_{1/2}$ of ∼2 weeks.

For the PEG−scFv conjugate **18** containing a stable linker (Scheme 4), 3-aminopropionaldehyde diethyl acetal was acylated with N_3−PEG_4−HSE **14**, followed by TFA cleavage of the acetal. Aldehyde **16** was used for reductive alkylation of DLX105, as described above, to provide **17** as a 62:35:3 mixture of unmodified−monoalkyl−dialkyl DLX105. Finally, the crude azido-linker−DLX105, **17**, was coupled by SPAAC to fluorescein−Lys(BCN)−$PEG_{40\ kDa}$, prepared by acylation of amino−$PEG_{40\ kDa}$ with fluorescein−Lys(BCN)−OSu,[4] and the product **18** was purified by ion-exchange fast protein liquid chromatography (FPLC). The inclusion of fluorescein in the conjugate enabled its detection in serum samples by high-performance liquid chromatography (HPLC) with fluorescence detection (below).

In Vitro Bioassay of PEG-DLX105 and Released DLX105 from Microsphere Conjugates. The stable PEG−DLX105 conjugate and the AP-DLX105 released from the microsphere−DLX105 conjugate were tested in a bioassay in which binding to TNFα neutralizes the TNFα-cytotoxicity of L929 cells (Figure 2).[13] Here, the PEG conjugate **18** showed an IC_{50} equipotent with free DLX105, demonstrating that PEGylated DLX105 binds tightly to TNFα. The AP-DLX105 **13** released by the microsphere−DLX105 conjugate after base treatment also gave the same IC_{50} as authentic DLX105, showing that AP-DLX105 binds TNFα as well as the parent DLX105 and that the process of coupling to and releasing from microspheres does not change TNFα binding and neutralizing ability.

Pharmacokinetic Studies. The C versus t plot for plasma DLX105 after intravenous (IV) injection in the rat is shown in Figure 3A and pharmacokinetic parameters after IV and subcutaneous (SC) injections are summarized in Table 1. There is a rapid initial α-distribution phase with $t_{1/2,\alpha}$ = 13 min, followed by an elimination phase with $t_{1/2,\beta}$ = 4.5 h. The high $V_{d,SS}$ of 0.85 to 1.5 L/kg for IV and SC injections, respectively, suggests that the tissue distribution volume is much larger than the 0.03 L/kg plasma volume in the rat.[14,15] Data indicated that the SC bioavailability of DLX105 was 100%.

Rats were injected IV with stable PEG−DLX105 and plasma PEG−DLX105 was analyzed by ELISA (Figure 3B) to show a $t_{1/2,\beta}$ of 51 h, about 11-fold longer than the $t_{1/2,\beta}$ of 4.5 h for the parent DLX105. An HPLC assay with fluorescence monitoring provided a $t_{1/2,\beta}$ of 55 h and confirmed the results from the ELISA. As observed with other PEGylated scFv fragments,[16] the $V_{d,SS}$ was significantly decreased, and the area under the curve (AUC) of PEG−DLX105 was significantly increased compared to the free scFv. The low $V_{d,SS}$ of 0.044 L/kg is slightly larger than the plasma volume and, together with the absence of a distribution phase, indicates that, in contrast to free scFv, PEG−DLX105 is largely confined to the vascular compartment.

Rats were also injected SC with the hydrogel microsphere−DLX105 conjugate and plasma samples measured for released AP-DLX105 by ELISA (Figure 3C). A small amount of immunoreactive material was released early, which is a result of unreacted DLX105 or **3** that was not removed during washing of the microspheres. Most of the released DLX105 showed $t_{1/2,\beta}$ of 270 h, some 60-fold longer than $t_{1/2,\beta}$ of 4.5 h for the parent DLX105 (Table 1). The released scFv is expected to have a $V_{d,SS}$ at least as high as the 1.5 L/kg determined for SC-injected DLX105; the value could be even higher if there is a slowly filled tissue compartment that requires prolonged exposure.

DISCUSSION

The objective of this work was to compare and evaluate two formats for half-life extension of antigen-binding mAb fragments. The first was a conventional stable PEG−scFv conjugate, **18**, containing the anti-TNFα scFv, DLX105; here,

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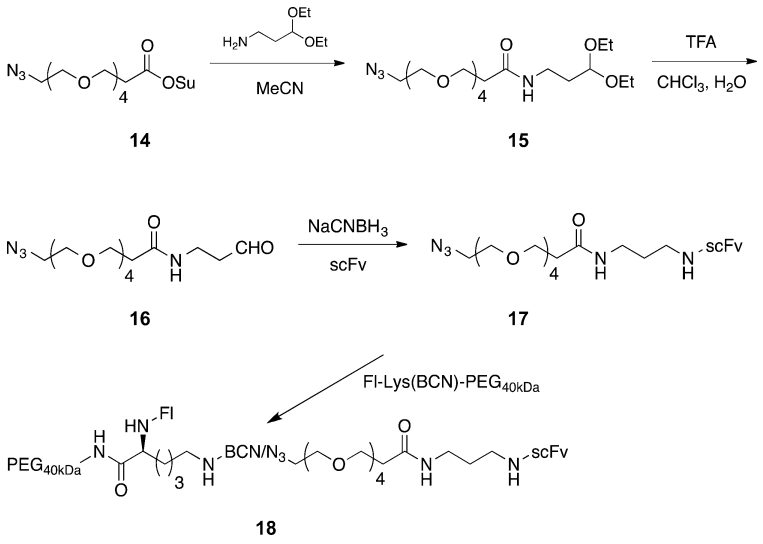
Scheme 4. Synthesis of Stable PEG-DLX105 Conjugate



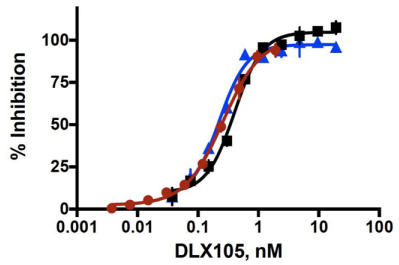


Figure 2. TNFα inhibitory bioassay of DLX105, PEG–DLX105, and AP-DLX105 after release from microspheres (pH 10; 20 h). IC_{50} values were 0.26 nM for DLX105 (black squares), 0.40 nM for PEG–DLX105 (red circles), and 0.22 nM for released AP-DLX105 (blue triangles).

the increase in hydrodynamic radius of ~9 nm afforded by the large 40 kDa four-branch PEG moiety[17] reduces the renal elimination of the covalently attached scFv. The second format was a releasable hydrogel microsphere–DLX105 conjugate, **12**, in which the scFv was attached to a noncirculating carrier by a self-cleaving β-eliminative linker; upon SC administration, the linker slowly cleaves and releases free AP-DLX105.

The PEG–DLX105 containing a stable linker was synthesized by conventional reductive alkylation of the amino terminus of DLX105 by an azido-linker aldehyde, followed by coupling the azide group to a cyclooctyne-modified $PEG_{40\ kDa}$. The PEG conjugate showed near-identical anti-TNFα activity as the free scFv and an ~12-fold increase in half-life in the rat, reflecting the slow renal elimination rate of the large $PEG_{40\ kDa}$–protein conjugate. The half-life extension achieved was similar to other site-specific PEG–scFv conjugates[16] and is exemplary of what can be achieved by PEGylation of proteins of this size. In humans, one might expect a $t_{1/2,\beta}$ of about 1 week for the stable PEGylated scFv.[18]

Recently, we described a drug delivery system in which 40 μm tetra-PEG hydrogel microspheres are covalently connected to a drug by a carbamate moiety via a self-cleaving β-eliminative linker;[8] upon SC administration through a small-bore needle, the drug is slowly released to the systemic circulation. Major advantages of this system are that (a) the released drug acquires a $t_{1/2,\beta}$ that is dictated by the $t_{1/2}$ of linker cleavage,[5,8] and (b) the in vivo linker cleavage rate is species-independent,[4,6] so the half-life extension in animal models is directly translatable to humans. Thus far, we have used the approach to prolong the $t_{1/2,\beta}$ of the 39 amino acid peptide exenatide[8] and the cyclic octapeptide octreotide.[9] However, both of these peptides are sufficiently small to allow site-specific carbamoylation by a linker succinimidyl carbonate at selected amino groups. Larger peptides and proteins usually contain multiple amino groups that make site-specific conjugation challenging.

Here, we describe a reductive alkylation approach for the selective attachment of β-eliminative linkers to the amino termini of larger proteins. The approach involved creating a linker that contained 3-aminopropionaldehyde at the scissile carbamate. The linker is attached to the α-amino group of a protein by reductive alkylation using stoichiometry that favors mono- versus multialkylation. The azido-linker protein is then attached by SPAAC to cyclooctyne-activated hydrogel microspheres using an orthogonal azide coupling group that was preinstalled in the linker. Upon β-eliminative cleavage of the linker carbamate, the aminopropyl moiety is transferred to the released protein. In this manner, we prepared a microsphere–scFv conjugate that had a predicted in vivo release rate and $t_{1/2,\beta}$ for the released protein of about 2 weeks.

When administered SC to rats, the scFv released from the microspheres had a $t_{1/2,\beta}$ of 270 h that, compared to that of SC injection of DLX105, represents a 60-fold half-life extension. Indeed, the $t_{1/2,\beta}$ of the released scFv is similar to the 9 to 14 day terminal half-lives of approved anti-TNFα mAbs in the human.[19] Increasing the plasma concentration of the released scFv can be simply achieved by increasing the dose of microsphere–scFv conjugate administered. A unique feature of the hydrogel delivery system is the ability to modify the half-life of the released drug simply by modifying the β-elimination rate of the linker. Thus, as recently described for a microsphere–octreotide conjugate,[9] by modifying the linker, it should be feasible to create a hydrogel microsphere–scFv conjugate that delivers a potent scFv over a period of a month or longer.

A major feature of small antibody fragments such as scFv is that they have higher tissue penetration than full-length mAbs.

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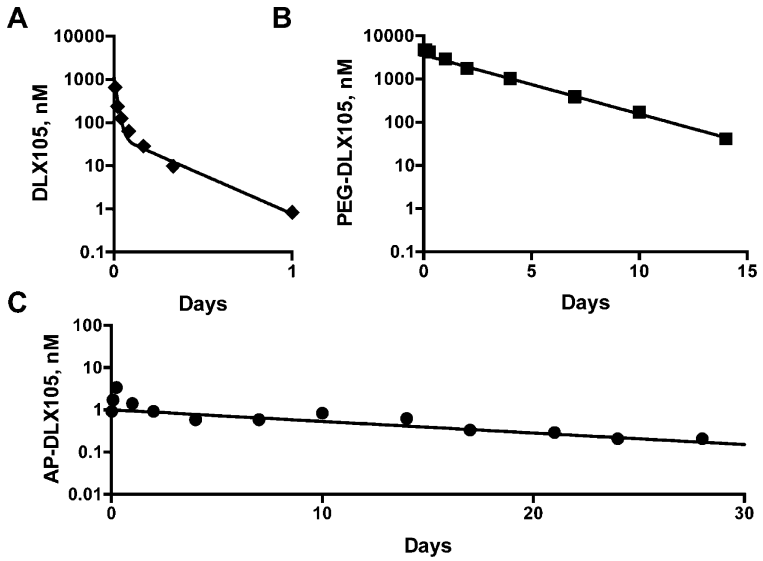

Figure 3. Pharmacokinetics of DLX105, PEG−DLX105, and AP-DLX105 released by microsphere−DLX105. Rat plasma C vs t curves of (A) DLX105 (7.5 mg/kg; ∼86 nmol/rat) administered IV (♦); (B) PEG−DLX105 (5 mg of conjugated DLX105/kg; ∼57 nmol DLX105/rat) administered IV (■); (C) AP-DLX105 released by SC-administered microsphere−DLX105 conjugate (5 mg of conjugated DLX105/kg; ∼57 nmol DLX105/rat) (●). Plasma levels of DLX105 were measured by ELISA using a rabbit polyclonal anti-DLX105 capture antibody, mouse anti-DLX105−goat HRP-anti-mouse detection system.

Table 1. Pharmacokinetic Parameters of DLX105, PEG−DLX105, and Microsphere (MS)−DLX105 Conjugate in the Rat

	DLX105		PEG−DLX105	MS−DLX105
administration	IV	SC	IV	SC
C_{max}, μM	1.2[a]	0.12	4.9[a]	0.0010
$t_{1/2,\alpha}$, hr	0.22	–[b]	–[b]	–[b]
$t_{1/2,\beta}$, hr	4.1	3.7	51	270
$V_{d,0}$, L/kg	0.23[c]	ND	0.039[c]	ND[f]
$V_{d,ss}$, L/kg	0.85[d]	1.5[d]	0.044[d]	≥1.5[e]
AUC_{inf}, μM-hr	0.82	0.84	310	0.46

[a]Extrapolated to C_0. [b]Insufficient early data to calculate. [c]Values for $V_{d,0}$ were calculated as dose/(C_0 × BW) (where BW is body weight). [d]$V_{d,ss}$ values were calculated as dose × MRT/(AUC × BW) (where MRT is mean residence time). [e]$V_{d,ss}$ assumed to be equal to or greater than SC-administered DLX105. All values are average determination of injected rodents, and standard errors are within ±11% of the mean. [f]ND indicates not determined.

Indeed, the distribution of an scFv in some tissues can exceed that of a mAb by as much as 100-fold,[1] and such differentials might be exploitable for tissue targeting. A rarely acknowledged disadvantage of using PEG, or other large carrier, to prolong the $t_{1/2,\beta}$ of a small antibody fragment is that the increased size results in greater confinement of the conjugate in the vascular compartment; this results in a decrease in $V_{d,SS}$[16] and a concomitant decrease in tissue distribution. Indeed, in the present case, the $V_{d,SS}$ of DLX105 decreased ∼20- to 30-fold fold upon PEGylation, showing the conjugate is confined within the vascular compartment and negating a major advantage of using a small antibody fragment. In contrast, the DLX105 released from the microsphere−DLX105 conjugate should have the same high tissue distribution as the SC injected scFv.

In summary, the cleavable hydrogel microsphere−scFv conjugate described here releases its protein cargo with a prolonged half-life of about 2 weeks, which is comparable to most full-length mAbs, and in a form that retains the high tissue distribution characteristic of small mAb fragments. Thus, whereas two or more injections per day might be necessary to maintain a free scFv at therapeutic concentrations, a hydrogel microsphere−scFv conjugate would require one or two injections per month. Clearly, other single or multiple antigen-binding antibody fragments should be amenable to the half-life extension approach described here.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.6b00469.

Additional details regarding the source of specialized materials; detailed synthetic and conjugation procedures; in vitro kinetic procedures; and in vivo pharmacokinetic methods, analyses, and simulations. (PDF)

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Notes

The authors declare the following competing financial interest(s): E.L.S., B.R.H., S.J.P., G.W.A., and D.V.S. are employees of and have equity in Prolynx. S.G., V.S., L.V., and T.J. are employees of Delenex.

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Supplementary Information

An approach for half-life extension of small antibody fragments that does not affect tissue uptake

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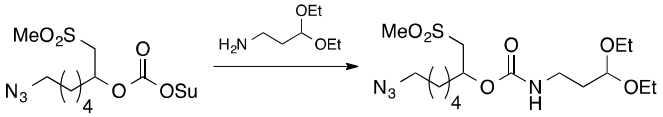
General

HPLC used a Shimadzu Prominence UFLC equipped with an SPD-M20A diode array detector and a RF-20A fluorescence detector. RP-HPLC analysis used a Jupiter 5µ C18 300A 150 x 4.6-mm HPLC column (Phenomenex) with a 1 min isocratic flow of 20% MeCN-0.1% TFA followed by a 10 min linear gradient of 20- to 100% MeCN-0.1% TFA at 1 mL min^{-1}. UV analyses were performed using a Hewlett-Packard 8453 UV-Vis or Thermo Scientific Nanodrop spectrophotometer. Amino-PEG reagents for microsphere preparation and 4-branched amino-PEG_{40kDa} for PEG conjugates were from JenKem. Succinimidyl 15-azido-4,7,10,13-tetraoxapentadecanoate (N_3-PEG_4 NHS ester) was from Broadpharm (BP-20518) and 1-amino-3,3-diethoxypropane was purchased from Sigma (A8597). *O*-[7-Azido-1-(methylsulfonyl)-2-heptyl]-*O*'-succinimidyl carbonate was prepared as previously reported [1]. DLX105 scFV was produced and purified as described [2]. Microspheres were measured as a packed/suspension/slurry with a density of 1 g/mL.

I. Releasable hydrogel microsphere-DLX105 conjugate.

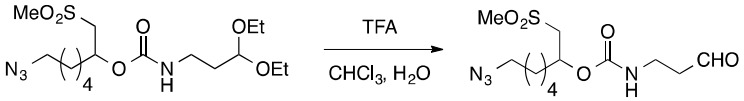
A. Synthesis of bifunctional releasable azido-linker aldehyde



8 **9**

7-Azido-1-(methylsulfonyl)-2-heptyl N-(3,3-diethoxypropyl) carbamate (**9**). 1-Amino-3,3-diethoxypropane (41 µL, 0.25 mmol) was added to a solution of *O*-[7-azido-1-(methylsulfonyl)-2-heptyl]-*O*'-succinimidyl carbonate **8** (43 mg, 0.11 mmol, 0.1 M final concentration) in 1.1 mL of MeCN. The reaction mixture was stirred at ambient temperature for 0.5 h, after which time the reaction was judged to be complete by TLC analysis. The mixture was partitioned between 40 mL of 1:1 EtOAc: $KHSO_4$ (5% aq). The aqueous phase was extracted with EtOAc (20 mL), and the combined organic extracts were successively washed with water, $NaHCO_3$ (sat aq), water, and brine (20 mL each). After drying over $MgSO_4$ and filtering, the crude EtOAc solution was concentrated to dryness to provide the title compound **9** (43 mg) as a crude colorless oil that was used in its entirety in the next step without further purification.

^{1}H NMR (300 MHz, $CDCl_3$) δ 5.32 (br t, *J*=5.1 Hz, 1H), 5.12 (quin, *J*=5.8 Hz, 1H), 4.55 (t, *J*=5.1 Hz, 1H), 3.66 (dq, *J*=8.9, 7.2 Hz, 2H), 4.49 (dq, *J*=9.0, 7.0 Hz, 2H), 3.21-3.37 (m, 5H), 3.12 (dd, *J*=14.7, 4.7 Hz, 1H), 2.97 (s, 3H), 1.82 (q, *J*=6.0 Hz, 2H), 1.75 (m, 2H), 1.59 (m, 2H), 1.40 (m, 4H), 1.20 (t, *J*=7.0 Hz, 6H).

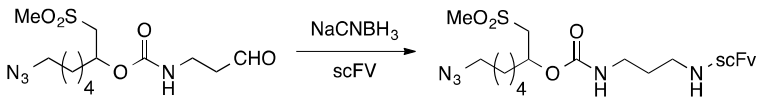


9 **10**

7-Azido-1-(methylsulfonyl)-2-heptyl N-(3-oxopropyl) carbamate (**10**). Water and TFA (0.18 mL each) were successively added to a solution of crude 7-azido-1-(methylsulfonyl)-2-heptyl *N*-(3,3-diethoxypropyl) carbamate **9** (43 mg) in 0.73 mL of $CHCl_3$. The reaction was vigorously stirred at ambient temperature for 2 h, after which time the reaction was judged to be complete by TLC analysis. The reaction mixture was concentrated to dryness by rotary evaporation, and the crude residue was purified by silica gel column chromatography (SiliaSep 4 g column) using a step-wise gradient of ethyl acetate in hexane: 0%, 25%, 45%, 55%, 65%, and 75% (30 mL each). Clean product containing fractions were combined and concentrated to provide the title compound **10** (17 mg, 51 µmol, 46% two steps) as a colorless oil.

^{1}H NMR (300 MHz, $CDCl_3$) δ 9.81 (s, 1H), 5.12-5.25 (m, 2H), 3.49 (q, *J*=5.9 Hz, 2H), 3.31 (dd, obscured, 1H), 3.27 (t, *J*=6.6 Hz, 2H), 3.13 (dd, *J*=14.7, 4.5 Hz, 1H), 2.98 (s, 3H), 2.77 (br t, *J*=5.2 Hz, 2H), 1.75 (m, 2H), 1.60 (m, 2H), 1.35-1.46 (m, 4H).

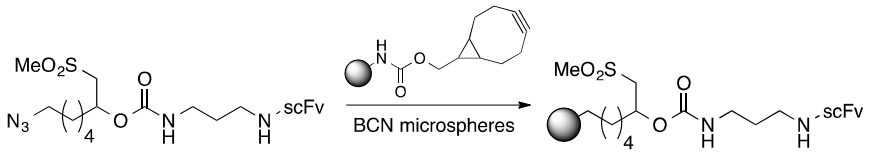
B. Preparation of hydrogel microsphere-DLX105 conjugate.



10 **11**

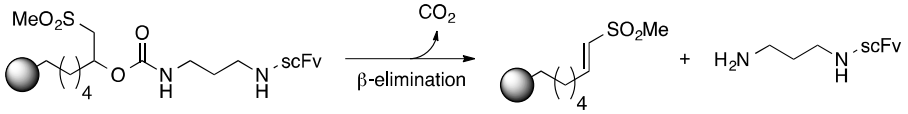
Reductive alkylation of DLX105 with a releasable azido linker. A solution of 55 mg DLX105 (2.1 µmol, final 200 µM) in 9.3 mL of 20 mM Na-Citrate, 115 mM NaCl, pH 6.0, was treated with 0.9 mg azido-aldehyde linker (Mod=$MeSO_2$) **10** (1.5 eq, 3.2 µmol, final 300 µM) in 95 µL MeCN, and 6.6 mg $NaCNBH_3$ (50 eq, 105 µmol, final 10 mM) in 1.05 mL of 20 mM Na-Citrate, 115 mM NaCl, pH 6.0, to give a final reaction volume of 10.5 mL. The reaction was kept at ambient temperature in the dark for 18 h, concentrated to 5 mL using an Amicon Ultra (10 kDa MW cutoff) concentrator and desalted on a PD-10 column equilibrated in 20 mM Na-Citrate, 115 mM NaCl, 0.05% (v/v) Tween-20, pH 6.0. A total of 44 mg protein (1.7 µmol, 80%) was recovered after the reaction and PD-10 purification. An aliquot (9 µL, 0.5 nmol, 12.6 µg) was treated with 1 µL of 5 mM (5 nmol) DBCO-PEG_{5kDa} and assayed for increases in molecular weight by SDS-PAGE. The PEG-shift assay showed the product to be a 62:35:3 mixture of unmodified: mono-alkyl: di-alkyl DLX105 to give 38% of protein in the final mixture that was either mono-alkyl or di-alkyl DLX105 (16.7 mg, 0.6 µmol).

Hydrogel $[BCN]_n$-microspheres. Amino microspheres containing crosslinking Mod=$(MeOEt)_2NSO_2$ were prepared and handled as reported [3], and derivatized with BCN-as previously reported [4].



11 **12**

Loading of $[BCN]_n$ microspheres with DLX105. The microsphere-BCN slurry (4.6 g, 12.5 µmol BCN) was placed into two 10 mL syringes [3] and exchanged into reaction buffer by washing five times with 6 mL of 20 mM NaCitrate, 115 mM NaCl, 0.05% (v/v) Tween-20, pH 6.0. A solution of the N_3-linker-DLX105 (Mod=$MeSO_2^-$, 7 mL, 636 nmol linker-scFv) in the same buffer was added to the microspheres, and the reaction was mixed by gentle end over end rotation at room temperature for 48 hrs. Reaction progress was followed by loss of absorbance in the supernatant at 280 nm. The final loading was determined by SDS-PAGE PEG-shift analysis of the remaining supernatant after treatment of 9 µL with 1 µL of 5 mM DBCO-PEG_{5kDa}, as described above. The final microsphere volume of **12** was 6 mL (6 g) and contained 630 nmol scFv.

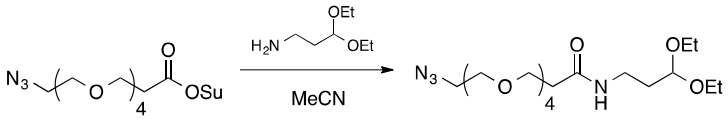


12 **13** (AP-scFv)

Microsphere release kinetics and loading. Kinetics of β-elimination were determined under accelerated release conditions using ~75 µL microsphere slurry/suspension **12** prepared above with addition of 450 µL of 100 mM Borate buffer 0.05% Tween 20 pH 9.4 at 37 °C in a 1.5 mL microcentrifuge tube (Eppendorf Protein LoBind). The tubes were sealed with Parafilm and incubated in a 37 °C water bath. At various times, the suspensions were mixed by inversion, centrifuged at 20,000 x g for 1 min, and the supernatant analyzed by by UV (Nanodrop) for released AP-ScFv for ΔA_{280}. The release rate (k_{obsd}) was calculated by fitting the released protein (A_{280}) vs time to the first-order rate equation. Assuming second-order rate constants are described by $k_2 = k_{obs}/[OH^-]$, k_{obs} values obtained at pH 9.4 were converted to k_{obs} values at pH 7.4. The total AP-scFV (**13**) released was determined from the absorbance after complete release (ε_{280}=50,000 M^{-1} cm^{-1}).

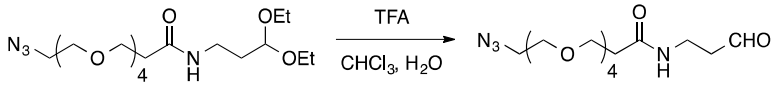
II. Synthesis of PEG-DLX105 (18)

A. Synthesis of bifunctional stable azido-linker aldehyde



14 **15**

N-(3,3-Diethoxypropyl)-15-azido-4,7,10,13-tetraoxapentadecanamide (**15**).
1-Amino-3,3-diethoxypropane (37 µL, 0.23 mmol) was added to a solution of succinimidyl 15-azido-4,7,10,13-tetraoxapentadecanoate **14** (41 mg, 0.11 mmol, 0.1 M final concentration) in 1.1 mL of MeCN. The reaction mixture was stirred at ambient temperature for 0.5 h, after which time the reaction was judged to be complete by TLC analysis. The mixture was partitioned between 40 mL of 1:1 EtOAc: $KHSO_4$ (5% aq). The aqueous phase was extracted with EtOAc (20 mL), and the combined organic extracts were successively washed with water, $NaHCO_3$ (sat aq), water, and brine (20 mL each). After drying over $MgSO_4$ and filtering, the crude EtOAc solution was concentrated to dryness to provide the title compound **15** (32 mg, 76 µmol max) as a crude colorless oil that was used in its entirety in the next step without further purification.

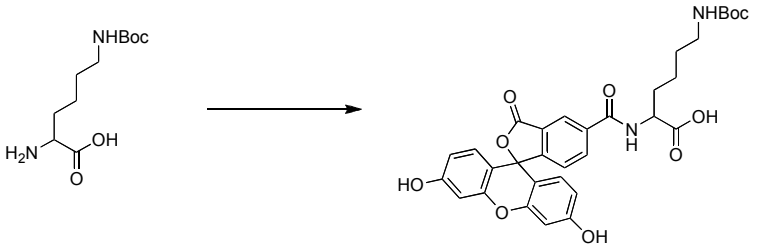


15 **16**

N-(3-Oxopropyl)-15-azido-4,7,10,13-tetraoxapentadecanamide (**16**). Water and TFA (0.13 mL each) were successively added to a solution of crude *N*-(3,3-diethoxypropyl)-15-azido-4,7,10,13-tetraoxapentadecanamide **15** (32 mg, 76 µmol max) in 0.51 mL of $CHCl_3$. The reaction was vigorously stirred at ambient temperature for 1.5 h, after which time the reaction was judged to be complete by TLC analysis. The reaction mixture was concentrated to dryness by rotary evaporation, and the crude residue was purified by silica gel column chromatography (SiliaSep 4 g column) using a step-wise gradient of acetone in CH_2Cl_2: 0%, 20%, 40%, 60%, and 80% (30 mL each). Clean product containing fractions were combined and concentrated to provide the title compound **16** (20 mg, 58 µmol, 53% two steps) as a pale yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$) δ 9.79 (s, 1H), 6.97 (br s, 1H), 3.62-3.74 (m, 16H), 3.54 (q, *J*=5.9 Hz, 2H), 3.40 (t, *J*=5.0 Hz, 2H), 2.73 (t, *J*=5.9 Hz, 2H), 2.47 (t, *J*=5.6 Hz, 2H).

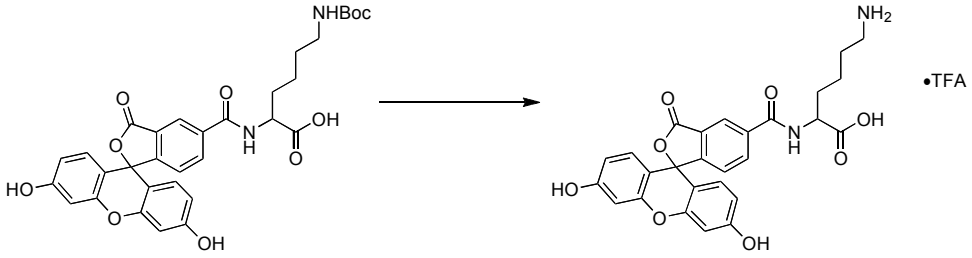
B. Synthesis of Fluorescein-Lys(BCN)-PEG$_{40kDa}$



Lys(Boc)OH **Fluorescein-Lys(Boc)OH**

Fluorescein-Lys(Boc)-OH. A 20-mL scintillation vial was charged with Lys(Boc)OH (12 mg, 49 µmol, 1.1 equiv) and 5-carboxy fluorescein NHS ester (20 mg, 42 µmol, 1.0 equiv). The solids were dissolved in 1:1 MeCN: 0.5 M aq $NaHCO_3$ (1 mL). The reaction mixture was stirred at ambient temperature for 2 h. C18 HPLC indicated the starting material had been consumed and replaced with a less polar product. The pH of the reaction mixture was adjusted to ~2 with 1 M HCl. The resulting yellow suspension was extracted with EtOAc (3 x 20 mL). The combined organics were washed with brine, dried over $MgSO_4$, filtered, and concentrated to an orange solid. Purification via C18 column chromatography (1 g column; load in 60% MeOH/H_2O; elute 0-75% MeOH/H_2O) afforded 16 mg (27 µmol, 65%) of the title compound as a yellow solid.

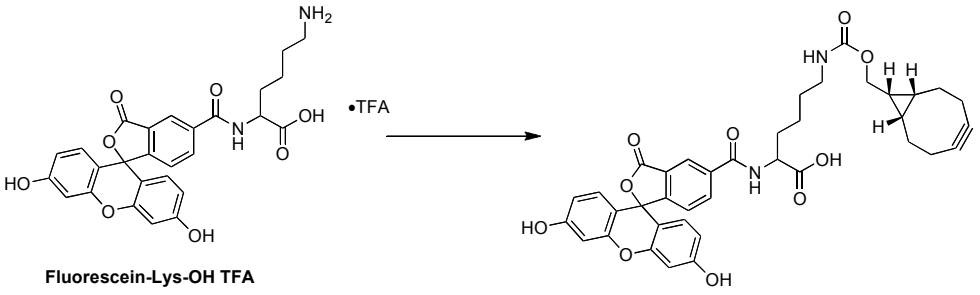
^{1}H NMR (CD_3OD, 300 MHz) δ 8.46 - 8.49 (m, 1 H), 8.23 (dd, J=8.1, 1.5 Hz, 1 H), 7.31 (dd, J=8.1, 0.8 Hz, 1 H), 6.69 (d, J=2.3 Hz, 2 H), 6.51 - 6.62 (m, 5 H), 4.63 (dd, J=9.2, 4.5 Hz, 1 H), 3.07 (t, J=6.4 Hz, 2 H), 1.81 - 2.10 (m, 2 H), 1.46 - 1.62 (m, 4 H), 1.40 (s, 9 H). C18 HPLC monitored at 441 nm: 95% (0-100% B 10 min, R_T = 9.02 min).



Fluorescein-Lys(Boc)OH **Fluorescein-Lys-OH TFA**

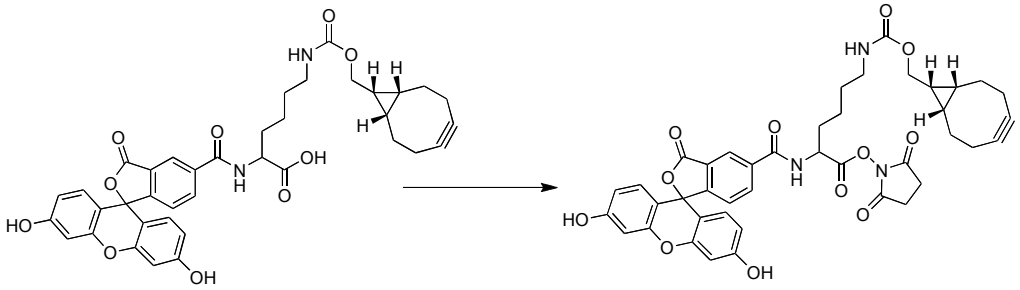
Fluorescein-Lys-OH•TFA. A 20-mL scintillation vial containing Fluorescein-Lys(Boc)OH (18 mg, 30 µmol, 1.0 equiv) was charged with CH_2Cl_2 (2 mL) and TFA (2 mL). The reaction mixture was stirred at ambient temperature for 30 min, and concentrated from toluene (3 x 5 mL) to afford an orange powder. This material was triturated with Et_2O (2 x 5 mL) and dried under high vacuum to afford 20 mg (quant) of the title compound as an orange powder.

^{1}H NMR (CD_3OD, 300 MHz) δ 8.48 (s, 1 H), 8.23 (dd, J=8.1, 1.7 Hz, 1 H), 7.31 (d, J=8.1 Hz, 1 H), 6.69 (d, J=2.1 Hz, 2 H), 6.49 - 6.62 (m, 5 H), 4.65 (dd, J=9.0, 4.7 Hz, 1 H), 2.96 (t, J=7.3 Hz, 2 H), 1.84 - 2.15 (m, 2 H), 1.67 - 1.82 (m, 2 H), 1.51 - 1.65 (m, 2 H). LRMS (ESI) m/z $[M+H]^+$ calcd for $C_{27}H_{24}N_2O_8$: 504.5; found: 505.6. C18 HPLC was monitored at 441 nm: 95% (0-100%B, R_T= 7.18 min).



Fluorescein-Lys(BCN)-OH. A 20-mL scintillation vial containing Fluorescein-Lys-OH•TFA (20 mg, 33 µmol, 1.1 equiv) and BCN-HSC (9 mg, 30 µmol, 1 equiv) was charged with DMF (1 mL) and 0.5 M aq. $NaHCO_3$ (0.5 mL; 125 mM $NaHCO_3$ final concentration). The reaction mixture was stirred at ambient temperature for 1 h. The reaction mixture was diluted with H_2O (15 mL) and the pH of the resulting solution was adjusted to ~2 with 1N aq HCl. Purification via C18 column chromatography (1 g Bond-Elute cartridge; gradient elution 10-100% MeOH/H_2O, pdt elutes ~50-60% MeOH/H_2O) afforded 17 mg of the title compound as a yellow oil.

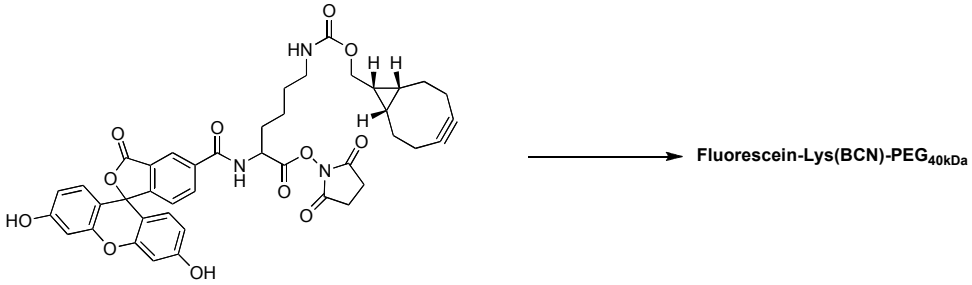
^{1}H NMR (CD_3OD, 300 MHz) δ 8.49 (s, 1 H), 8.24 (dd, J=7.9, 1.3 Hz, 1 H), 7.31 (d, J=8.1 Hz, 1 H), 6.69 (d, J=2.4 Hz, 2 H), 6.60 (d, J=8.7 Hz, 2 H), 6.54 (dd, J=8.7, 2.4 Hz, 2 H), 4.62 (dd, J=9.4, 4.7 Hz, 1 H), 4.10 (d, J=8.1 Hz, 1 H), 3.10 - 3.17 (m, 2 H), 2.07 - 2.30 (m, 7 H), 1.82 - 1.97 (m, 1 H), 1.43 - 1.67 (m, 7 H), 1.25 - 1.41 (m, 2 H), 0.80 - 0.96 (m, 2 H). LRMS (ESI) m/z $[M+H]^+$ calcd for $C_{38}H_{36}N_2O_{10}$: 680.2; found: 681.7. C18 HPLC monitored at 441 nm: 96% (0-100% B 10 min, R_T = 9.38 min). This material was dissolved in 5:1 MeCN:MeOH (6 mL total) and fluorescein content was determined by A_{495} (100 mM borate, pH 10) ε = 80,000 M^{-1} cm^{-1}: theoretical: 5.00 mM; actual 3.45 mM (21 µmol, 70% yield).



Fluorescein-Lys(BCN)-HSE. A 4-mL vial was containing Fluorescein-Lys(BCN)-OH (14 mg, 21 µmol, 1.0 equiv) was charged with a solution of N-hydroxy succinimide (46 mM in THF, 0.48 mL, 22 µmol, 2.05 equiv). The mixture was stirred at ambient temperature for 5 min then a solution of dicyclohexylcarbodiimide (DCC) (44 mM in THF, 0.5 mL, 22 µmol, 1.05 equiv) was added. The reaction mixture was allowed to stand at 4 ˚C for 18 h during which time a fine white precipitate formed. The reaction mixture was filtered through a pipette equipped with a small plug of cotton with the aid of THF (2 mL). The resulting filtrate was concentrated to an oil and immediately redissolved in ~1 mL of MeCN. Fluorescein content of this solution was determined by A_{495} (100 mM borate, pH 10) ε = 80,000 M^{-1} cm^{-1}: 16.0 ± 0.1 mM. This solution of the title compound was used immediately in the next step without further purification.

An aliquot (10 µL) of this solution was quenched in a solution of methoxyethyl amine (10 mM in MeCN). LRMS (ESI) m/z $[M+H]^+$ calcd for $C_{41}H_{43}N_3O_{10}$: 738.3; found: 738.8. C18 HPLC was

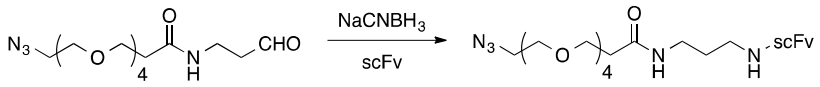
monitored at 441 nm: 97% (aliquot quenched with 10 mM methoxyethyl amine in MeCN, 0-100% B, R_T = 7.98 min).



Fluorescein-Lys(BCN)-HSE

Fluorescein-Lys(BCN)-PEG$_{40kDa}$. A 4-mL vial was charged with 4-branched PEG$_{40kDa,}$-NH$_2$ (300 mg, 7.5 µmol 1.0 equiv, 7.1 mM final concentration) and was dissolved in MeCN (0.5 mL). A solution of Fluorescein-Lys(BCN)-HSE (16 mM in MeCN, 0.56 mL, 9.0 µmol, 1.2 equiv, 8.5 mM final concentration) and iPr_2NEt (4 µL, 23 µmol, 3 equiv, 23 mM final concentration) were added. The reaction mixture was stirred at ambient temperature for 2 h. The reaction mixture was dialyzed against 50% MeOH/H_2O (0.8 L) for 16 h and against 100% MeOH (0.9L) for 5 h. The retentate was concentrated to a thick oil and the desired product was precipitated by the addition of MTBE (150 mL). The resulting solids were collected by vacuum filtration through a sintered glass funnel and air dried to afford 250 mg of the title compound as an orange powder. This powder was immediately dissolved in MeCN (2 mL, volumetric flask). Fluorescein content was determined by $A_{495\ nm}$ using ε = 80,000 M^{-1} cm^{-1}: theoretical 3.60 mM; found 2.24 mM (4.5 µmol, 62%). C18 HPLC was monitored at 441 nm: 95% (20-100%B, R_T = 7.74 min).

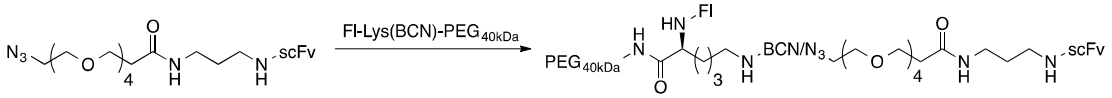
C. Reductive alkylation of DLX105 with a stable azido-linker-aldehyde



16 **17**

Reductive alkylation of DLX105. A solution of 55 mg DLX105 (2.1 µmol, final 200 µM) in 9.3 mL of 20 mM Na-Citrate, 115 mM NaCl, pH 6.0, was treated with 0.8 mg stable azido-aldehyde linker **16** (1.5 eq, 3.2 µmol, final 300 µM) in 95 µL MeCN, and 6.6 mg $NaCNBH_3$ (50 eq, 105 µmol, final 10 mM) in 1.05 mL of 20 mM Na-Citrate, 115 mM NaCl, pH 6.0, to give a final reaction volume of 10.45 mL. The reaction was kept at ambient temperature in the dark for 18 h, concentrated to 5 mL using an Amicon Ultra (10 kDa MW cutoff) concentrator and desalted on a PD-10 column equilibrated in 20 mM Na-Citrate, 115 mM NaCl, 0.05% (v/v) Tween-20, pH 6.0. 50.4 mg protein (1.9 µmol, 92% recovery) was recovered after the reaction and PD-10 purification. A purified reaction aliquot was treated with DBCO-PEG$_{5kDa}$ and assayed for a shift in molecular weight by SDS-PAGE. The PEG-shift assay showed the product to be a 62:35:3 mixture of unmodified: mono-alkyl: di-alkyl DLX105. Linker modified protein was 38% of the final mixture (19.2 mg, 0.7 µmol). Of the total azido-DLX105 (mono-alkyl and di-alkyl, 92% was mono-alkyl **17**.

D. PEG-DLX105 conjugate preparation



17 **18**

PEG-DLX105 conjugate (**18**). A solution containing *Fluorescein-Lys(BCN)-PEG$_{40kDa}$* (114 nmol BCN) in MeCN was placed in a 2 mL reaction vial and dried under vacuum. The dried PEG was then solubilized by addition of 500 µL of a solution of DLX105-linker-N_3 **17** (104 nmol) in 20 mM Na Citrate, 115 mM NaCl, pH 6.0 and thereby also initiating the SPAAC reaction. The reaction was left overnight at ambient temperature with gentle end-over-end mixing. The final reaction progress was determined by SDS-PAGE analysis of the reaction using the PEG shift assay described above. The reaction mixture was purified by ion exchange chromatography on an AKTA purifier with a 1 mL HighTrap SP FF column (GE Healhthcare) using a 0 to 20% B gradient over 20 minutes at a 1 ml/min flow rate (Buffer A: 20 mM MES pH 6.0, Buffer B: 20 mM MES,1 M NaCl pH 6.0)**.** Peaks were collected and analyzed by SDS-PAGE. The unreacted **17** bound to the column, whereas the PEG-moiety of **18** apparently shielded the charge of the protein and prevented binding. Fractions containing the PEG-DLX105 conjugate compound **18** eluted in the void volume and were concentrated and buffer exchanged into 20 mM sodium citrate with 115 mM sodium chloride, pH 6.0 using an Amicon Ultra 10 kDa MW cutoff concentrator to a final volume of 500 µL.

III. In vitro TNFα binding bioassay of PEG-DLX105 (18) and released AP-DLX105 from MS-conjugates (13).

DLX105-microspheres **12** (1.4 mg DLX105/g of slurry) were diluted to 0.1 mg/mL of DLX105 in 20 mM Glycine buffer, pH 10, and incubated for 20 h at 37°C with slight agitation to release DLX105 into the supernatant. The slurry was centrifuged 10 min at 16,100 x g, after which the supernatant was again centrifugated. The DLX105 concentration in the supernatant was determined by UV measurement at 280 nm (ε_{280}=50000 $M^{-1}cm^{-1}$). PEG-DLX105 **18** was diluted from a 6.8 mg DLX105/mL stock solution in 20 mM sodium citrate with 115 mM sodium chloride, pH 6.0.

TNFα binding was determined by a cytotoxicity-neutralizing assay [5]. All test and reference samples were serially diluted in assay medium (RPMI 1640 without phenolred (Gibco, #32404-014), GlutaMAX (Gibco, #35050-038), 5 % FCS (Gibco, #10270-106), Pen Strep (Penicillin Streptomycin; (+) 5.00 Unit/mL Pen, (+) 5.00 µg/mL Strep; Gibco, #15070-063)) with TNFα (100 pg/mL) and Actinomycin (1 µg/mL) to appropriate concentrations and added to microtiter wells containing 1.5 to 2 x 10^4 cells/well (L929 murine fibroblast cells ATCC® #CCL-1™). After 20 hours of incubation at 37°C, 5% CO_2, the number of living cells was assessed by an XTT Cell proliferation kit (Roche, product #11465015001). The absorbance in each well was measured at 492 nm with background reference measured at 680 nm. The resulting titration curves were fit to a 4-parameter nonlinear equation and IC_{50} values were determined using GraphPad Prism software.

IV. Pharmacokinetics of DLX105 conjugates in rats.

A. In vivo administration.

DLX105. Dosing solutions were prepared in 20 mM sodium citrate containing 115 mM sodium chloride, pH 6.0 at 10 mg/mL scFv. Three male Sprague Dawley rats, weight ~300g, were dosed either i.v. or s.c. at 7.5 mg DLX105/kg rat (~2.25 mg scFv/rat). Blood samples (200-300

µL) were drawn at 0, 0.2, 0.5, 1, 2, 4, 8, and 24 hours and added to 0.1 vol of 1M Na citrate 0.1% Pluoronic F48 pH 4.5 to lower the sample pH and provide plasma.

*PEG-DLX105 conjugate **18***. Dosing solutions of 4-branch PEG-DLX105 (**18**) were prepared in 20 mM sodium citrate containing 115 mM sodium chloride, pH 6.0 at 190 µM conjugate (5 mg/mL scFv). Three male Sprague Dawley rats weighing ~300g each were dosed i.v. at 5 mg DLX105/kg (1.5 mg DLX105/rat). Blood samples (200-300 µL) were drawn at 0, 0.5, 2, 6, 24, 48, 120, 168, 240, 336 hours and added to 0.1 vol of 1M Na citrate, 0.1% Pluoronic F48, pH 4.5 [6], to provide acidified plasma.

*DLX105-microspheres **12***. Syringes (1 mL Leur-Lock, BD) were filled under sterile conditions with the scFv-microsphere slurry (**12**) in 50 mM NaP_i 0.04% Tween 20, pH 6.2 (Mod=$MeSO_2^-$, 1.4 mg DLX105/g slurry). The content of each prefilled syringe was administered s.c. in the flank of cannulated male Sprague Dawley rats, average weight ~300g. The syringe/needle assembly was purged with the microspheres and weighed prior to and following dosing in order to determine the mass of the slurry delivered to each rat (average dose 1.1 mg slurry containing 1.5 mg DLX105). Blood samples (200-300 µL) were drawn at 0, 1, 2, 4, 8, 24, 48, 72, 120, 168, 240, 336, 432, 504, 600, 672 hours, the plasma was collected as above and the samples were frozen at -80 °C until analysis.

B. Plasma analysis of DLX105 and PEG-DLX105.

Soluble 40kDa 4-branch PEG conjugates, HPLC. Plasma samples were thawed on ice and diluted 1:4 in 10 mM MES pH 6.0 (running buffer A) (15 µL + 45 µL buffer). Standards were prepared from the PEG-DLX105 (**18**) at 10 µM in rat plasma, diluted 1:4 in running buffer A as above, and serially diluted 3-fold in 25% rat plasma/75% running buffer A. Sample analysis was performed with 20 µL injections onto a Shimadzu Prominence HPLC equipped with a low pressure Fast Flow SP HP column (GE Healthcare) at 40 °C. Sample elution was with the following step gradient at 1 mL min^{-1}. Following injection, the column was washed with 100% buffer A for 5 minutes followed by 100% buffer B (10 mM MES, 1 M NaCl pH 6.0) for 5 minutes. The HPLC was equipped with a post column mixer delivering 1M ethanolamine pH 10.0 at 0.1 mL min^{-1} to increase the pH for fluorescein detection followed by two detectors, an SPD-M20A diode array detector and an RF-20A fluorescence detector measuring fluorescence at excitation 494 nm and emission 521 nm. Peak areas for the standards (PEG-DLX105 RV = 7.4 mL) were subject to a linear fit and used to calculate the concentration and PEG-fluorescein-scFv in the sample injections.

Sandwich-ELISA of DLX105, AP-DLX105 and PEG-DLX105. An unlabeled rabbit polyclonal anti-DLX105 antibody was coated on microplates at a concentration of 0.5 µg/mL (produced at Genscript). Before each incubation, the plates were washed three times with PBS containing 0.05% Tween. After blocking, plasma samples and PEG-DLX105 or unmodified DLX105 standards were submitted to ELISA at different dilutions (1:20 to 1:12,500). PEG-DLX105, DLX105 and AP-DLX105 were quantitated by a murine monoclonal anti-DLX105 antibody (produced at Eurogentec) and by a goat anti-mouse IgG-HRP (Sourthern Biotech #103105). The ELISA was developed with BM Blue POD substrate, and the absorbance was measured at 450 nm. The results were evaluated by comparison to standard curves of PEG-DLX105 or DLX105 using the SoftMax Pro Software.

C. Pharmacokinetic analysis

Pharmacokinetic analyses were performed using Prism software, fitting appropriate single (PEG-DLX105, MS-DLX105) or double exponential (DLX105) equations. The data was analyzed by a least squares fit with weighting by $1/SD^2$.

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