SUPPLEMENTAL INFORMATION

Site-specific conjugation of single domain antibodies to liposomes enhances photosensitizer uptake and photodynamic therapy efficacy

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Experimental

Chemicals and reagents

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol (Chol), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG-Mal, average PEG molecular mass of 2000 amu), 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*n*-glycero-3-phosphocholine (NBD-PC), L-α-phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (Rho-PE) were from Avanti Polar Lipids (Alabaster, AL). Ampicillin, cysteine (Cys), cysteamine (CA), sodium 2-sulfanylethanesulfonate (MESNA), β-mercaptoethanol (BME), chloroform, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Coomassie Brilliant Blue, tris(2-carboxyethyl)phosphine (TCEP), bovine serum albumin (BSA), glycerol, Bromophenol Blue, $NaPO_4$, sodium dodecyl sulfate (SDS), DNAse, lysozyme, zinc phthalocyanine (ZnPC), cOmplete (protease inhibitors), and pyridine were from Sigma-Aldrich (St. Louis, MO). Isopropyl β-D-1-thiogalactopyranoside (IPTG) and Ni-nitrilotriacetic acid (Ni-NTA) beads were from Thermo Fisher Scientific (Waltham, MA). Tetracycline was obtained from OPG Farma (BUVA, Uitgeest, the Netherlands). PBS was from Lonza (Basel, Switzerland). Bugbuster, $CaCl_2$, tris-HCL, and NaCl were from Merck KGaA (Darmstadt, Germany). PEG10k-maleimide (PEGmal) was from JenKem Technology (Allen, TX).

Lipids were dissolved in chloroform and stored at -20 °C in the dark under a nitrogen atmosphere. The concentration of the DPPC and DSPE-PEG stocks was determined by an inorganic phosphate assay [1]. ZnPC was dissolved in pyridine at 178-µM concentration and stored at room temperature in the dark.

Plasmids and expression of anti-EGFR sdAbs

The fusion gene encoding the antagonistic anti-EGFR sdAb EGa1 (described previously by *Hofman et al.* [2]) was fused to a C-terminal intein domain and elastin-like polypeptide (intein-ELP, kind gift from Carlos Filipe, McMaster University, Hamilton, Ontario, Canada). Briefly, the EGa1 sdAb gene in pTXB1 (kind gift from William Leenders, Radboud University, Nijmegen, the Netherlands) was cloned into the intein-ELP construct by using the sites XbaI and BsiWI. Subsequently, an enterokinase cleavage site was introduced between the leaded sequence and sdAb domain to remove the non-processed leader sequence after protein expression. There was a 6× His tag between the sdAb and the intein domain, and between the intein and ELP domain for visualization and purification purposes.

Escherichia coli BLR(DE3) cells (New England Biolabs, Ipswich, MA) were transformed with the adapted pTXB1 construct and selected using 100 µg/mL ampicillin and 12.5 µg/mL tetracycline. The expression of the fusion protein (comprising sdAb-intein-ELP) was performed by inducing cultures between OD_{600} = 0.5-0.6 with 0.3 mM IPTG for 20 h at 25 °C. The harvested cultures were lysed in 1:50 bugbuster supplemented with lysozyme, DNAse and protease inhibitors (Roche; cOmplete), and incubated for 15 min at room temperature (RT). The supernatant was collected by centrifugation at 19,000 × g at 4 °C and incubated with in PBS-equilibrated Ni-NTA beads (1 mL beads per L culture) for 1 h at 4 °C. The beads were washed three times with PBS, after which the fusion protein was eluted in 250 mM imidazole in PBS.

For the VHH cleavage experiment, the original pTXB1 construct (lacking the ELP) was used, as the high salt concentrations used for the ELP precipitation did not allow subsequent SDS-PAGE analysis of the cleavage products. The fusion protein (sdAb-intein-CBD) was

expressed, loaded onto chitin beads (New England Biolabs) and incubated with 64 mM CA, MESNA, or BME at RT. After 1, 4 and 21 hours at 4°C, samples were taken and 1:1 diluted in 2x non-reducing Laemmli buffer. Samples were analyzed following separation by 15% SDS-PAGE, blotting on nitrocellulose membrane (Bio-Rad, Hercules, CA), and detection with monoclonal anti-polyHis-peroxidase antibody (A7058, Sigma-Aldrich). The bands were quantified in ImageJ (National Institutes of Health, Bethesda, MD).

For all subsequent experiments, the fusion protein in the lysates of the transformed *E. coli* cultures (*Plasmids and expression of anti-EGFR sdAbs*) was precipitated by addition of NaCl to a final concentration of 2 M. The protein pellet was resuspended in ice-cold cleavage buffer (20 mM $NaPO_4$, pH = 7.0, and either 250 mM CA and 250 mM MESNA and incubated overnight at 4 °C. The salt concentration of the mixture was again raised to 2 M to precipitate the cleaved intein-ELP domains at 37 °C. After centrifugation (19,000 × g, 10 min, 40 °C), the supernatant was dialyzed against PBS, supplemented with 2 mM $CaCl_2$, and incubated with enterokinase (New England Biolabs) overnight at 37 °C to completely remove the leader sequence. After centrifugation (19,000 × g, 10 min, RT), the supernatant containing the thiol-functionalized sdAb (sdAb-CA or sdAb-MESNA) was further purified by size exclusion chromatography using a PBS-equilibrated Superdex 75 10/300 GL column (GE Healthcare, Munich, Germany). The purified protein was stored at 4 °C until further use.

Table S1. Dissociation kinetics of the sdAb from the intein-ELP fusion protein induced by MESNA, CA, and BME (positive control). Rate constant *k* and plateau data were obtained with one-phase exponential decay fitting, of which the goodness of fit (R^2) is also provided (for method see "*Statistical analysis*"). The plateau data are expressed as percentage relative to BME. There were no significant differences between the best fit parameters.

Thiolating agent	One-phase exponential decay fit		
	k	*Plateau*	R^2
CA	1.818	28.69	0.9899
MESNA	2.285 **	98.24	0.9863
BME	~6.271	~275.2	0.8752

SdAb-fluorophore conjugation

The sdAbs in PBS (approximately 4-8 mg/mL) were reduced by the addition of 5 mM TCEP for 5 min at RT, desalted against PBS using Zeba Spin Desalting columns (Thermo Fisher Scientific), and incubated with 1 or 4 molar equivalents of NHS-IRDye800 or Mal-IRDye800 (both from Li-Cor Biosciences, Lincoln, NE) overnight at RT. The samples were desalted against PBS as described above, also to remove unbound fluorophore. The samples were diluted (1:1, v/v) in 2× Laemmli sample buffer without DTT, separated by SDS-PAGE, stained with Coomassie Brilliant Blue, and detected with an Odyssey CLx imager (Li-COR Biosciences). The formation of sdAb-fluorophore complexes is shown in Fig. S1. Based on these results, and in order to keep the reactions comparable in terms of conjugation and uptake efficiencies, subsequent experiments were performed with 4 molar equivalents of IRDye800.

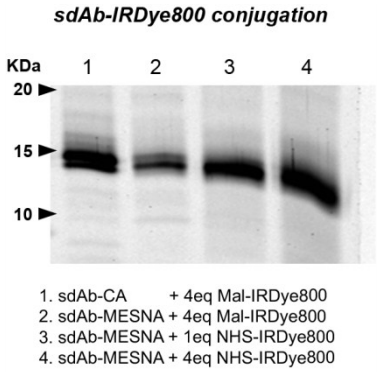

Figure S1. Fluorescence emission from sdAb proteins indicates successful labeling of sdAb-CA and sdAb-MESNA to Mal-IRDye800 and NHS-IRDye800. Both proteins were labeled with 4 molar equivalents of Mal-IRDye800 (lanes 1 and 2). Additionally, sdAb-MESNA was also randomly labeled with 1 or 4 molar equivalents NHS-IRDye800 (lanes 3 and 4). After SDS-PAGE, the fluorescent signal per sdAb was imaged.

Cell culture

The human epidermoid carcinoma cell line A431 and mouse fibroblast cell line NIH 3T3 2.2 ('3T3 2.2') were obtained from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco / Invitrogen, Carlsbad, CA) supplemented with 8% fetal bovine serum (FBS) (v/v). The cell culture medium was supplemented with antibiotics to a final concentration 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine (all from PAA Laboratories, Cölbe, Germany). All cell lines were kept in culture at 37 °C in a humidified atmosphere containing 5 % CO_2. Cells were typically subcultured once a week at a 1:10 ratio. All incubations with sdAb-IRDye conjugates or liposomes were performed with serum and phenol red-free DMEM (SPRF-DMEM).

SdAb-IRDye800 binding affinity assessment

A431 cells were seeded in 96-wells plates at 8 × 10^4 cells/well and grown to confluence over 48 h. Cells were washed with SPRF-DMEM (4 °C) and incubated for 30 min at 4 °C. A concentration range of sdAb-IRDye800 diluted in SPRF-DMEM was added to the cells. After incubation for 90 min at 4 °C, cells were washed thrice with SPRF-DMEM

to remove unbound sdAb-IRDye800. Bound sdAb-IRDye was detected using the Odyssey CLx scanner and corrected for background prior to statistical analysis.

Table S2. SdAb binding kinetics to A431 cells under non-internalizing conditions (4 °C). Data were obtained from one site binding hyperbola fitting of the data. Asterisks indicate significant differences with the sdAb-CA + Mal-IRDye800 group (see "*Statistical analysis*").

sdAb + Dye variants	Single site binding hyperbola fit		
	Bmax	*Kd*	R^2
sdAb-CA + Mal-IRDye800	1.818	28.69	0.9899
sdAb-MESNA + NHS-IRDye800	2.285	98.24 **	0.9863
sdAb-CA + NHS-IRDye800	~6.271	~275.2	0.8752
sdAb-MESNA + Mal-IRDye800	0.223	30.24	0.8041

Preparation of liposomes

ZnPC-encapsulating liposomes (non-targeted liposomes, NTLs) were prepared as described previously [3] according to the lipid film hydration technique [4]. Lipids in chloroform were premixed at DPPC:chol:DSPE-PEG-Mal molar ratios of 77:15:8, respectively, and ZnPC was added to the lipid mixture at a ZnPC:lipid ratio of 0.003. To fluorescently label the liposomes, Rho-PE (fluorescence spectroscopy) or NBD-PC (confocal laser scanning microscopy) were added to the lipid mixture at the expense of DPPC at 0.2 and 5 mol%, respectively. The lipids were desiccated under nitrogen gas, after which the lipid film was vacuum-exsiccated for 30 min. Subsequently, the lipid film was hydrated with physiological buffer composed of 0.88 % (w/v) NaCl, 10 mM HEPES, pH = 7.4, 0.292 osmol/kg. Liposomes were sized by brief sonication and extrusion through 0.2-µm aluminum oxide filters (Whatman, GE Healthcare, Little Chalfont, UK).

For sdAb coupling, the C-terminal thiol on the sdAbs was reduced by 5 mM TCEP for 5 min at RT, desalted against PBS using Zeba Spin desalting columns (Thermo Fisher Scientific), and incubated with liposomes for 1 h at RT in the dark followed by incubation at 4 °C overnight in the dark. The sdAb:total lipid ratios used were 10 µmol/mmol. The non-reacted maleimide groups on DSPE-PEG-Mal were quenched by addition of BME at equimolar concentration (relative to the DSPE-PEG-Mal) and incubated for 1 h at RT. The non-coupled sdAbs and BME were removed by 2 × ultracentrifugation at 2 × 10^5 ×g for 40 min at 4 °C (Fig. S2), after which the total lipid concentration was determined as described in "*Chemicals and reagents*." Liposomes were analyzed for size and polydispersity index (PDI) by photon correlation spectroscopy (Table S3) using a Zetasizer 3000 (Malvern Instruments, Malvern, UK) as described previously[3]. Liposomes were stored in the dark at 4 °C and under a nitrogen atmosphere until further use.

For analysis of purity and conjugation efficiency, liposomes were loaded onto 15% SDS-PAGE gels and separated under reducing conditions. The sdAb was visualized using Coomassie Brilliant Blue stain and detected using the Odyssey CLx scanner.

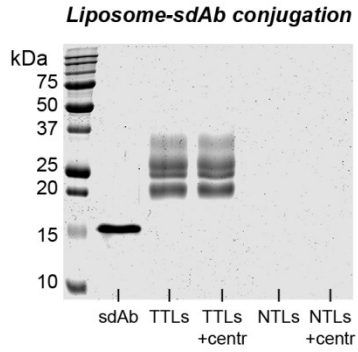

Figure S2. SDS-PAGE analysis to confirm the coupling of liposomes containing DSPE-PEG-Mal with sdAb-CA (TTLs). Unconjugated sdAb has a size of 15.75 kDa (lane 1), whereas SdAb coupled to liposomal DSPE-PEG-Mal caused a size increase of ~5kDa. The unbound sdAb-CA was removed by dual ultracentrifugation ("+centr", lane 3), although most of the protein was efficiently conjugated to the liposomes (lane 2). Liposomes not incubated with sdAb-CA (NTLs-) were included as negative controls (lanes 4 and 5).

Table S3. (Immunotargeted) liposome composition and characterization by photon correlation spectroscopy.

Name	Formulation (molar ratio)	sdAb per mM lipid	Size [nm]	PDI
NTLs	DPPC:Chol:DSPE-PEG (66:30:4)	-	88.4 ± 3.2	0.21 ± 0.06
TTLs	DPPC:Chol:DSPE-PEG-Mal (66:30:4)	10 µmol	134.1 ± 4.2	0.26 ± 0.08

Determination of liposomal uptake

For the liposomal uptake studies, A431 and 3T3 2.2 cells were seeded in 96-wells plates at a density of 2 × 10^3 cells/well and cultured for 24 h. Next, cells were washed once with SPRF-DMEM and incubated with 125 µM TTLs or NTLs that were fluorescently labeled with Rho-PE (described in "*Preparation of liposomes*"). Cells were incubated for 48 h at standard culture conditions and subsequently washed twice with SPRF-DMEM. Rho fluorescence was measured on a FluoStar Optima fluorescent plate reader (BMG Labtech, Ortenberg, Germany). Data were corrected for background fluorescence of untreated cells.

For the ZnPC uptake studies, A431 cells were seeded in 96-wells plates at a density of 1.6 × 10^4 cells/well and cultured for 24 h. Next, cells were washed

once with SPRF-DMEM and incubated with ZnPC containing TTLs or NTLs in SPRF-DMEM (liposome concentrations indicated separately per experiment) for 1 h under standard culture conditions. After incubation, the cells were washed twice with DMEM and once with 2 mM $NaPO_4$ buffer, pH = 7.0, after which the plates were vacuum-exsiccated for 15 min to remove residual fluid. The fluorescence intensity of ZnPC was detected at 670 nm (Odyssey CLx scanner) and quantified using Image Studio 3.1 (Li-Cor Biosciences). Data were corrected for background fluorescence. It should be noted that Rho-labeled liposomes exerted no toxicity in A431 and 3T3 2.2 cells (Fig. S3) as determined using the Alamar Blue method (section "*Cell viability assay*").

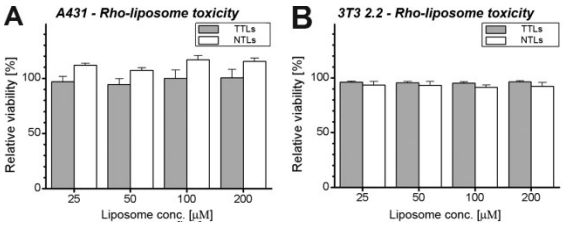

Figure S3. (A) Viability of A431 cells exposed to increasing concentrations of Rho-TTLs and Rho-NTLs. (B) Viability of 3T3 2.2 cells exposed to increasing concentrations of Rho-TTLs and Rho-NTLs. All data represent mean ± standard deviation of N = 6. Statistics were performed using a one-way ANOVA and Sidak's post hoc test. No statistically significant differences were found.

Cell viability assay

Viability assays using Alamar Blue reagent were performed according to the manufacturer's protocol (AbD Serotec, Oxford, UK). Briefly, cells were incubated for 2 h with SPRF-DMEM containing 1:10 (v/v) Alamar Blue (Life Technologies). The conversion of the Alamar Blue to its fluorescent analogue (λ_{ex} = 560 nm, λ_{em} = 590 nm) was measured with a FluoStar Optima fluorescence plate reader at 590 ± 40nm (BMG Labtech). Cell viability was expressed as a percentage of dead cells relative to untreated cells after background subtraction.

Photodynamic therapy

For the PDT experiments, cells were seeded in 96-wells plates (Greiner Bio-One, Kremsmünster Austria) at a density of 4 × 10^3 cells/well and cultured for 24 h. Next, cells were incubated for 10 min with ZnPC-TTLs or ZnPC-NTLs (125 µM total lipid concentration, including cholesterol) in medium, washed twice with SPRF-DMEM, and kept at standard culture conditions for 2 h to facilitate internalization of the liposomes. Cells were subsequently treated with PDT. All plates were subjected to a light dose of 10 J/cm^2 at 670 ± 10 nm during 42 min (1 LED per well, 4 mW/cm^2 as measured with an Orion Laser power/energy monitor, Ophir Optronics, Jerusalem, Israel). After PDT, the plates were immediately returned to the incubator. Cell viability was assayed after 24 h.

Confocal laser scanning microscopy

The uptake specificity of ZnPC-containing liposomes and intracellular distribution of ZnPC were investigated. Microscope cover slips (24 × 40 mm, VWR, Lutterworth, UK) were placed in 6-wells plates and coated with 5×10^{-4}% (w/v) fibronectin in 1 mL of sterile 0.9% NaCl solution (Fresenius Kabi, Bad Homburg, Germany) for 2 h at 37 °C prior to cell seeding. A431 cells and 3T3 2.2 cells were seeded on the fibronectin-coated microscope coverslips in 6-wells plates at 2.0 × 10^5 cells/mL. Cells reached subconfluence during overnight incubation. The cells were exposed to ZnPC-TTLs labeled with or without sdAb for 10 min, after which cells were washed twice in PBS and received fresh medium. Cells were cultured for 3.5 h, after which cells received fresh SPRF-DMEM supplemented with 50 nM MitoTracker Red CMX-ROS (Molecular Probes / Life Technologies, Eugene, OR). After 30 min of incubation, cells were washed twice with PBS and the cell-containing coverslips were mounted on microscope slides with VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Slides were dried for 1 h and sealed with nail polish. Confocal laser scanning microscopy was performed on a Leica SP8 confocal microscope system (Leica Microsystems, Wetzlar, Germany). Cells were analyzed for fluorescence of 4', 6'-diamidino-2-phenylindole (DAPI) (λ_{ex} = 405 nm, λ_{em} = 415-500 nm), MitoTracker Red CMX-ROS (λ_{ex} = 579 nm, λ_{em} = 589-700 nm), and ZnPC (λ_{ex} = 660 nm, λ_{em} = 670-750 nm). Images were processed in Leica Application Software using the Advanced Fluorescence module (Leica Microsystems). An additional close-up image of the intracellular ZnPC levels after TTL incubation is shown in Fig. S4.

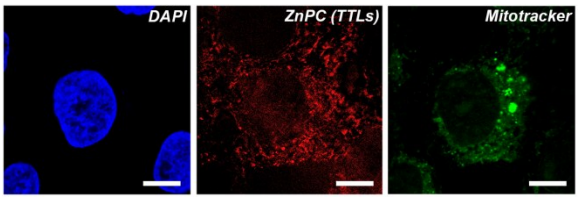

Figure S4. Confocal laser scanning microscopy images of A431 cells incubated with TTLs. Cells were stained with DAPI (nucleus, blue) and Mitotracker Red (green). ZnPC is displayed in red. All images were obtained using a 63x oil immersion objective and digital zoom. Scale bar = 5 µm.

Statistical analysis

Statistical analyses and curve fitting were performed in GraphPad Prism (GraphPad Software, San Diego, CA). Data were analyzed for normality using a Kolmogorov-Smirnov test. Normally distributed data sets were analyzed with a

one-way ANOVA and subsequent Sidak's post-hoc test for multiple comparisons. Non-Gaussian data sets were analyzed using a Kruskal-Wallis test and a Dunn's post-hoc test for multiple comparisons. All data are reported as mean ± standard deviation. In the figures, differences between cells treated with TTLs and NTLs are indicated with (*) and differences between similarly treated cell lines are indicated with (#). The level of significance is reflected by a single ($p < 0.05$), double ($p < 0.01$), triple ($p < 0.005$), or quadruple sign ($p < 0.001$). Best fit parameters between data sets were compared using an extra sum-of-squares F-test.

References

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