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**Chapter 4**

**Nanobody-functionalized liposomes enhance photosensitizer uptake and photodynamic therapy efficacy**

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**Abstract**

Photodynamic therapy (PDT) is a non-invasive treatment modality based on the activation of a tumor-replete photosensitizer for the production of reactive oxygen species (ROS) and subsequent induction of tumor cell death. However, currently used photosensitizers do not specifically accumulate in tumor tissue, causing reduced treatment efficacy and adverse events such as skin phototoxicity. The encapsulation of photosensitizers into a liposomal drug delivery system has previously been shown to be beneficial for the treatment outcome. Tumor-cell targeted liposomal drug delivery systems may further improve treatment outcome by increasing the accumulation in and uptake by tumor cells. This paper presents a biochemical engineering approach for the development of photosensitizer-containing tumor cell targeted liposomes (TTLs) that may be used to enhance the efficacy of PDT. Single-domain antibodies (sdAbs) that recognized the human epidermal growth factor receptor (EGFR) were expressed as intein-fusion proteins and functionalized with a C-terminal thiol group upon by cysteamine-induced cleavage of the sdAb section. Immunotargeted liposomes were subsequently obtained by the inclusion of maleimide-functionalized poly-ethylene glycol on the liposome surface, and the conjugation of the sdAb to the liposomes via cysteine-maleimide interactions. These TTLs demonstrated efficient uptake kinetics and tumor killing efficacy when used for PDT on human EGFR-overexpressing A431 tumor cells. The nanobodies and encapsulated drugs used in this study are interchangeable and can thus be used in a variety of targeted drug delivery applications.

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**1. Introduction**

Photodynamic therapy (PDT) is a non-to-minimally invasive treatment modality for superficial solid tumors. PDT entails the topical, oral, or systemic administration of a photosensitizing agent, accumulation of the photosensitizer in the tumor, and subsequent irradiation of the tumor with high-power resonant light to excite the photosensitizer ¹. The excited photosensitizer transfers its
energy to molecular oxygen (O₂), yielding singlet oxygen (¹O₂) in the process. Alternatively, excited photosensitizers transfer their excited electrons to O₂ to produce superoxide anion (O₂⁻). Both types of reactive oxygen species (ROS) are capable of oxidizing biomolecules and, when produced excessively such as during PDT, induce lethal oxidative stress in light-exposed cancer cells ⁵. Most systemically injected photosensitizers localize to the tumor vasculature (endothelium) and are taken up by perivascular tumor cells ³. As such, PDT causes cellular demise, shutdown of the tumor vasculature, and an anti-tumor immune response, altogether resulting in removal of the tumor ⁴.

Despite the notable therapeutic efficacy with respect to superficial tumors such as skin-, head and neck-, and esophageal cancers, there are several factors that have restricted the implementation of PDT for cancers that are recalcitrant to, or ineligible for standard therapy. First, patients have to remain shielded from light for up to 12 weeks (in case of Photofrin) due to skin photosensitivity ⁵, ⁶, which stems from the profound accumulation of (orally or systemically administered) photosensitizer in the skin. As a result, several treatment centers have discontinued the use of PDT for terminally ill patients inasmuch as forcing these patients to remain inside for an extended period of time is unethical. Second, most of the currently approved photosensitizers have suboptimal photophysical and photochemical properties, as a result of which the treatment outcomes are suboptimal, particularly in larger tumors ⁵.

Accordingly, we have previously explored the feasibility of encapsulating zinc-phthalocyanine (ZnPC) into a liposomal formulation intended for passive but specific targeting towards the tumor tissue ⁷ by exploiting the enhanced permeability and retention effect of tumors ⁸. The experiments demonstrated that ZnPC-containing liposomes are photodynamically active and were capable of inducing tumor cell death, despite limited uptake. However, liposomes that are actively targeted for tumor cell-specific uptake have also shown great promise for targeted cancer therapies ⁹-¹¹, including PDT ¹². Immunotargeted liposomes are typically decorated with whole antibodies (150 kDa) or fractions thereof (Fab fragments, 55 kDa) that may substantially influence the size and in vivo pharmacokinetics and disposition of the liposomes (e.g., effector functions mediated by the Fc domain)¹³. Moreover, these antibodies are relatively unstable, are expensive to obtain, and are not easily manipulated for biochemical engineering purposes ¹⁴. To circumvent the drawbacks of using whole antibodies, the heavy chain-only antibodies found in camels and primitive fish species are an interesting alternative. The epitope-recognition domains of these antibodies are composed of a single immunoglobulin termed VHH or nanobody. These nanobodies are highly stable, can refold after denaturation, are highly soluble, small (15 kDa), and relatively easy manipulated for (site-specific) conjugation to drug carriers (reviewed in ¹⁵). Moreover, they are easily obtained and engineered via microbial expression.

The conjugation of drugs or reporter molecules to nanobodies has traditionally been performed by random coupling to lysine residues. Nanobodies bear 4-6 lysine residues on average that can be modified by N-hydroxysuccimidine-based cross-linking ¹⁶. Compared to monoclonal antibodies, the relative large surface area of nanobodies involved in antigen recognition renders this approach less suitable. The labeling of lysine(s) in one of the complementarity determining regions (CDRs) can be catastrophic for antigen binding. In addition, this random conjugation leads to a heterogeneous population and complicates further downstream processing and applications. Thus, a new strategy in which the conjugation of nanobodies to liposomes is more tightly controlled would greatly benefit the development of tumor cell-targeted liposomes (TTLs) for cancer therapy.

Site-specific conjugation of nanobodies is possible by fusing its C-terminus to an intein domain. Intein domains are naturally expressed within proteins to repress their activity, but can be spliced from the polypeptide chain under specific conditions ¹⁷. Self-excision releases the intein domain and restores the function of the host protein by fusing the splice-ends ¹⁷. This self-excision process can be exploited for site-specific modification of the N- or C-terminus of nanobodies via expressed protein ligation (EPL) ¹⁸. Since the N-terminus is in close proximity to the CDRs, it is potentially involved in antigen binding and thus not suitable for the conjugation of biomolecules. The C-terminus of nanobodies, however, is rarely involved and can be used for EPL. First, the native peptide bond is converted to a thioester, after which a Cys (or analog) can be ligated to restore the native
peptide bond (also known as native chemical ligation (NCL))\textsuperscript{18,19}. Subsequently, the native peptide bond may be used for further functionalization.

This study focuses on the development of TTLs that deliver the photosensitizer zinc phthalo-cyanine (ZnPC) specifically towards EGFR-overexpressing tumor cells by controlled coupling of anti-EGFR nanobodies to the liposome surface\textsuperscript{20,21}. We optimized the EPL technology to generate nanobodies that contain a free thiol at the C-terminus, which allowed subsequent formation of TTLs via maleimide-based chemistry. The specific uptake of TTLs by EGFR-overexpressing cancer cells (A431 cell line) was investigated, after which \textit{in vitro} proof-of-concept was demonstrated with respect to PDT efficacy.

2. Materials and methods

2.1 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-PEGMAL, average PEG molecular mass of 2000 amu), 1-palmitoyl-2-{[\((7\text{-nitro-2-1,3-benzoazadizol-4-yl)}\) amino]hexanoyl}-sn-glycero-3-phosphocholine (NBD-PC), L-\(\alpha\)-phosphatidylethanolamine-N-{lissamine rhodamine B sulfonyl} (Rho-PE) were from Avanti Polar Lipids (Alabaster, AL). Ampicillin, Cys, cysteamine (CA), sodium 2-sulfanylthanesulfonate (MESNA), \(\beta\)-mercaptopoethanol (BME), chloroform, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), coomassie brilliant blue, tris(2-carboxyethyl)phosphine (TCEP), bovine serum albumin (BSA), glycerol, bromophenol blue, NaPO4, sodium dodecyl sulfate (SDS), DNAse, lysozyme, ZnPC, and pyridine were from Sigma-Aldrich (St. Louis, MO). Isopropyl \(\beta\)-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, CaCl2, tris-HCL, and NaCl were from Merck KGaA (Darmstadt, Germany). PEG10k-maleimide (PEGmal) was from JenKem Technology (Allen, TX).

All lipid stocks 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 were determined by an inorganic phosphate determination\textsuperscript{23}. ZnPC was dissolved in pyridine at 178-μM concentration and stored at 4 °C in the dark.

2.2 Plasmids and expression of anti-EGFR nanobodies

The antagonistic anti-EGFR nanobody EGa1 (described previously Roovers \textit{et al.}\textsuperscript{20}) was fused to a C-terminal intein domain and elastin-like polypeptide (intein-ELP, kind gift from Carlos Filipe, McMaster University, Canada). Briefly, the EGa1 nanobody 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. There was a 6 × His tag between the nanobody and the intein domain for visualization and purification purposes. \textit{Escherichia coli} BLR(DE3) cells 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 was optimized by inducing cultures between OD600 = 0.5-0.6 with 0.3 mM IPTG for 2, 4, or 20 hrs at 25, 30, or 37 °C. The harvested cultures were lysed in 1:50 bugbuster supplemented with lysozyme and DNase and incubated for 15 min at room temperature (RT). The supernatant was collected by centrifugation at 19,000 \(\times\) g at 4 °C and incubated 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. Fusion protein in the collected fractions was precipitated by addition of NaCl to a final concentration of 2 M. The intein-ELP domains were subsequently cleaved from the nanobody with CA. The protein pellet was resuspended in ice-cold cleavage buffer (20 mM NaPO4, pH = 7.0, 250 mM CA) 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. The supernatant was dialyzed against PBS, supplemented with 2 mM CaCl₂, and incubated with enterokinase (NEB) overnight at 37 °C to completely remove the leader sequence. The supernatant containing the thiol-functionalized nanobody 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. Sample analysis was performed by separation with 15% SDS-PAGE, blotting on nitrocellulose membrane (Bio-Rad, Hercules, CA), detected using monoclonal anti-polyHis-peroxidase antibody (A7058, Sigma-Aldrich) and quantified using ImageJ 1.47 (National Institutes of Health, Bethesda, MD).

2.3 Protein conjugation analysis

The nanobody conjugation to DSPE-PEGmal was assayed prior to liposome preparation. DSPE-PEGmal (in chloroform) was dried under vacuum followed by addition of the nanobody and incubation for 20 h at RT. The protein/lipid samples were subsequently separated by reducing 15% SDS-PAGE gel to analyze the attachment of PEGmal and DSPE-PEGmal. The protein was visualized using coomassie brilliant blue stain, detected with an Odyssey CLx scanner (Li-Cor Biotechnology, Bad Homburg, Germany) and quantified by densitometric analysis using ImageJ.

2.4 Preparation of liposomes

ZnPc-encapsulating liposomes were prepared as described previously 7 according to the lipid film hydration technique 22. Lipids in chloroform were premixed at DPPC:chol:DSPE-PEGmal 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 or NBD-PC 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, United Kingdom).

The C-terminal thiol on the nanobodies were 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 nanobody:total lipid ratios used were 2 or 10 nmol/μmol. The non-reacted maleimide groups on DSPE-PEGmal were quenched by addition of BME at equimolar concentration (relative to the DSPE-PEGmal) and incubated for 1 h at RT. The non-coupled nanobodies and BME were removed by 2 × ultracentrifugation at 200,000 × g for 40 min at 4 °C, after which the total lipid concentration was determined as described in section 2.1. Liposomes were analyzed for size and polydispersity index by dynamic light scattering using the Zetasizer 3000 (Malvern Instruments, Malvern, UK) as described previously 7, and stored in the dark at 4 °C and under a nitrogen atmosphere until further use. When indicated, liposomes were prepared with only 20% of the nanobodies on the liposomal surface (termed `TTLs+1/5VHH'), which were employed to determine the concentration-dependent effect of nanobodies on size and PDT efficacy.

2.5 Cell culture

The human epidermoid carcinoma cell line A431 (CRL-1555) and mouse fibroblast cell line NIH 3T3 2.2 (‘3T3 2.2’) were 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% CO₂. Cells were typically subcultured once a week at a 1:15 ratio.
2.6 Photodynamic therapy

For the PDT optimization experiments, cells were seeded in 96-wells plates (Greiner Bio-One, Kremsmünster Austria) at a density of $4 \times 10^3$ cells/well and cultured for 24 h. Next, cells were incubated for 10 min with ZnPC-TTLs (125 μM total lipid concentration, including cholesterol) in medium, washed twice with DMEM without phenol red (DMEM-PR), and treated by PDT. For the remaining experiments, cells were seeded in 96-wells plates at a density of $2 \times 10^3$ cells/well and cultured for 24 h. In the time course experiment, cells were incubated for 10 or 30 min with 125 μM ZnPC-TTLs at the indicated time points, washed twice with DMEM-PR, and treated by PDT. In the 30-min exposure experiments, cells were cultured for 22 h and incubated for 30 min with 62.5, 125, or 250 μM ZnPC-TTLs, washed twice with DMEM-PR, cultured for 1.5 h (to facilitate internalization of the liposomes), and treated by PDT. In other experiments, cells were cultured for 22 h and incubated for 10 min with 125 μM ZnPC-TTLs (unless mentioned otherwise), washed twice with DMEM-PR, cultured for 2 h (to facilitate internalization of the liposomes), and treated by PDT. For PDT treatment, all plates were subjected to a light dose of 10 J/cm² at 670 ± 10 nm during 42 min (1 LED per well, 4-mW/cm² 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 the next day (section 2.7).

2.7 Cell viability assay

Viability assays using Alamar Blue reagent were performed according to the manufacturer’s protocol (AbD Serotec, Oxford, United Kingdom). Briefly, cells were incubated for 2 h with phenol red-lacking but otherwise fully supplemented DMEM (DMEM-PR) containing 1:10 (v/v) Alamar Blue (Life Technologies). The conversion of the Alamar Blue to its fluorescent analogue ($\lambda_{ex} = 560$ nm, $\lambda_{em} = 590$ nm) was measured with a FluoStar Optima fluorescence plate reader at 590 ± 40nm (BMG Labtech, Ortenberg, Germany). Cell viability was expressed as a percentage of dead cells relative to untreated cells after background subtraction.

2.8 Determination of liposomal uptake

For the uptake studies, A431 and 3T3 2.2 cells were seeded in 96-wells plates at a density of $1.6 \times 10^4$ cells/well and cultured for 24 h. Next, cells were washed once with phenol red-lacking but otherwise fully supplemented DMEM (DMEM-PR) and incubated with ZnPC-TTLs in medium (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 NaPO₄ 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 Biotechnology, Lincoln, Nebraska).

2.9 Confocal laser scanning microscopy

To investigate TTL uptake dynamics, cells were stained with transferrin. A431 cells seeded at $5 \times 10^4$ cells/mL and grown on gelatin (0.1% in MQ)-coated coverslips for 2 d. Cells were incubated with 125 μM ZnPC-TTLs (total lipid concentration) for 30 min at 37 °C, washed once with DMEM and chased in DMEM for 110 min at 37 °C. Subsequently, Alexa Fluor 488-conjugated transferrin (AF-transferrin, Alexa Fluor 488 from Life Technologies) was incubated for 10 min at 37 °C, after which the plates were washed twice with ice-cold PBS and fixed with 4 % formaldehyde (w/v) at 4 °C. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Roche, Basel, Switzerland) and images were acquired using a Zeiss LSM 700 inverted confocal microscope (Zeiss, Oberkochen, Germany). Cells were analyzed for fluorescence of DAPI ($\lambda_{ex} = 405$ nm, $\lambda_{em} = 445 \pm 30$ nm), AF-transferrin ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 530 \pm 30$ nm), and rhodamine-labeled TTLs (rho-TTLs) ($\lambda_{ex} = 561$ nm, $\lambda_{em} = 581 \pm$
Next, the uptake specificity of ZnPC-TTLs 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^{-6} g (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 × 10^4 cells/mL. Cells reached subconfluence during overnight incubation. The cells were exposed to ZnPC-TTLs labeled with or without nanobody 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 serum-free 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 DAPI (λex = 405 nm, λem = 415-500 nm), MitoTracker Red CMX-ROS (λex = 579 nm, λem = 589-700 nm), and ZnPC (λex = 660 nm, λem = 670-750 nm). Images were processed in Leica Application Software using the Advanced Fluorescence module (Leica Microsystems).

2.10 Statistical analysis

Statistical analyses were performed in GraphPad Prism (GraphPad Software, San Diego, CA). Unless indicated otherwise, data was 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+VHH and TTLs-VHH 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).

3. Results

3.1 Expression and purification of anti-EGFR nanobodies

A step-by-step description for the derivation, functionalization, and ligation of VHH nanobodies to liposomes is provided in Fig. 1. The construct that was cloned into E. coli BLR(DE3) contained a gene that encoded a fusion protein consisting of the anti-EGFR VHH, a C-terminal intein domain, and an ELP. In order to optimize the yield of the fusion protein (step 1), the bacterial cultures were incubated at different temperatures and time intervals and analyzed for protein yield. Typical yields are provided in Fig. 2A, showing that the highest yield (set at 100%) was obtained after culturing the cells at 25 °C or 30 °C for 20 h. In contrast, protein production was only 58% of the maximum yield upon incubation at 37 °C. To determine whether this was caused by premature cleavage of the intein domain, the amount of free VHH was quantified. No free VHH could be detected after 2 h, while the amount of free VHH after 20 h was identical (set at 100%) for all temperatures (Fig. 2B). However, there was a temperature-dependent increase in free VHH after 4 h. Free VHH levels were < 2%, 8%, and 12% for 25 °C, 30 °C, and 37 °C, respectively. These results indicate that expression at 25 °C for 20 h was most optimal and that proteins expressed at 37 °C were probably more prone to degradation by proteases, causing a loss in the total fusion protein yield.

The thiol-mediated cleavage of intein-fusion proteins (Fig. 1, steps 3 and 4) was evaluated for several thiol compounds. Of these, Cys and CA can perform both intein cleavage (expressed protein ligation) and native chemical ligation (Fig. 1 steps 3 and 4) in one reaction. MESNA (2-sulfanylthanesulfate) and 2-mercaptoethanol (BME) can only perform EPL since these compounds lack
an amino group and thus allow the ligation of Cys-bearing peptides. The EPL was most efficient with BME, followed by MESNA and CA, and slowest with Cys (data not shown). Despite the slower onset, the total intein cleavage by CA after 21 h was comparable to MESNA. However, since CA also mediates the NCL, we explored the potential for CA to introduce a reactive thiol group at the C-terminus of nanobodies. Thiol-mediated cleavage with 250 mM CA efficiently produced a VHH bearing a C-terminal thiol group (VHH-CA), as shown in Fig. 2C, lane 1. There were some minor contaminations, probably due to degradation of the intein-ELP domain, but these were effectively removed by sequential size exclusion chromatography (Fig. 2C, lanes 2-6).

3.2 Conjugation of anti-EGFR nanobodies to liposomes

To confirm the presence and assess the reactivity of the C-terminal thiol group, we incubated VHH-CA with increasing concentrations of DSPE-PEGmal and analyzed the protein and protein-lipid complex migration on SDS-PAGE gel (Fig. 2D). The unmodified VHH-CA migrated at approximately 15 kDa, corresponding to the molecular weight of a single VHH-CA molecule (15.76 kDa). The conjugation of VHH-CA to DSPE-PEGmal was confirmed by a band shift towards higher molecular weights (~22 kDa). The band density increased up to 5 equivalents DSPE-PEGmal in a concentration-dependent manner, signifying an increasing degree of conjugation. Next to labeling of VHH-CA with a single DSPE-PEGmal molecule, multiple modifications were visible. The incubation with 2.5 equivalents DSPE-PEGmal yielded the highest conversion rate of a single modified VHH-CA.
with a minimum extent of multiple modifications. The conjugation of VHH-CA to liposomes was also confirmed by SDS-PAGE analysis (Fig. 2E). Only a minor fraction of uncoupled VHH-CA was detected after conjugation, which was removed by ultracentrifugation (not shown, only visible after adjusting the brightness/contrast of the image). Despite the preferential conjugation to the reactive thiol (band at ~22 kDa), random labeling was also observed. This was probably the result of the excess of DSPE-PEGmal, which first reacted with the thiol group and subsequently with lysine residues on the surface of VHH-CA. TTLs bearing no VHH typically had a size of 88 nm, whereas TTLs+VHH were substantially larger at 134 nm (Fig. 2F). TTLs bearing only 1/5 of the total VHH load were smaller (108 nm) but exhibited a higher PDI, indicating greater particle size heterogeneity.

### 3.3 Binding and uptake of TTLs

The association between fluorescently (Rho-)labeled TTLs+VHH and TTLs-VHH and cells was measured in EGFR-overexpressing A431 cells and EGFR-null 3T3 2.2 cells. As shown in Fig. 3A, there was a dose-dependent increase in Rho fluorescence when A431 cells were incubated for 48 h with Rho-TTLs, regardless of the presence of VHH. However, the fluorescence intensity of A431 cells incubated with 50 and 200 μM TTLs+VHH was significantly higher compared to A431 cells incubated with equimolar concentrations of TTLs-VHH. With respect to the specificity of association, 3T3 2.2 cells exhibited substantially less affinity for and/or internalization of TTLs, regardless of the presence (80%) with a minimum extent of multiple modifications. The conjugation of VHH-CA to liposomes was also confirmed by SDS-PAGE analysis (Fig. 2E). Only a minor fraction of uncoupled VHH-CA was detected after conjugation, which was removed by ultracentrifugation (not shown, only visible after adjusting the brightness/contrast of the image). Despite the preferential conjugation to the reactive thiol (band at ~22 kDa), random labeling was also observed. This was probably the result of the excess of DSPE-PEGmal, which first reacted with the thiol group and subsequently with lysine residues on the surface of VHH-CA. TTLs bearing no VHH typically had a size of 88 nm, whereas TTLs+VHH were substantially larger at 134 nm (Fig. 2F). TTLs bearing only 1/5 of the total VHH load were smaller (108 nm) but exhibited a higher PDI, indicating greater particle size heterogeneity.

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### 3.3 Binding and uptake of TTLs

The association between fluorescently (Rho-)labeled TTLs+VHH and TTLs-VHH and cells was measured in EGFR-overexpressing A431 cells and EGFR-null 3T3 2.2 cells. As shown in Fig. 3A, there was a dose-dependent increase in Rho fluorescence when A431 cells were incubated for 48 h with Rho-TTLs, regardless of the presence of VHH. However, the fluorescence intensity of A431 cells incubated with 50 and 200 μM TTLs+VHH was significantly higher compared to A431 cells incubated with equimolar concentrations of TTLs-VHH. With respect to the specificity of association, 3T3 2.2 cells exhibited substantially less affinity for and/or internalization of TTLs, regardless of the presence (80%) with a minimum extent of multiple modifications. The conjugation of VHH-CA to liposomes was also confirmed by SDS-PAGE analysis (Fig. 2E). Only a minor fraction of uncoupled VHH-CA was detected after conjugation, which was removed by ultracentrifugation (not shown, only visible after adjusting the brightness/contrast of the image). Despite the preferential conjugation to the reactive thiol (band at ~22 kDa), random labeling was also observed. This was probably the result of the excess of DSPE-PEGmal, which first reacted with the thiol group and subsequently with lysine residues on the surface of VHH-CA. TTLs bearing no VHH typically had a size of 88 nm, whereas TTLs+VHH were substantially larger at 134 nm (Fig. 2F). TTLs bearing only 1/5 of the total VHH load were smaller (108 nm) but exhibited a higher PDI, indicating greater particle size heterogeneity.
ence of VHH (Fig. 3B). These results indicate that TTLs+VHH effectively and specifically associated with cells that overexpress the target receptor. Moreover, TTLs+VHH were effective in the delivery of ZnPC to the tumor cells, as is shown in Fig. 3C. All concentrations >15.6 μM TTL+VHH showed a significant increase in intracellular ZnPC after a 48 h incubation compared to TTLs-VHH.

### 3.4 Intracellular localization of TTLs and TTL-delivered ZnPC

The intracellular distribution of ZnPC following TTL delivery was visualized by confocal microscopy. Fig.4A demonstrates that Rho-TTLs-VHH (red) are not effectively internalized by A431 cells, whereas Rho-TTLs+VHH were readily internalized (Fig. 4B). Cells were co-stained with AF-transferrin, which is rapidly internalized via receptor-mediated endocytosis. Green fluorescent loci therefore correspond to early endosomes. No clear colocalization of transferrin and Rho was observed, indicating that the Rho-labeled lipids were not present in early endosomes. This, in combination with the granular staining pattern of Rho-phospholipids, suggests that the liposomes were present in late endosomes and/or lysosomes. Although the intracellular localization of liposomal lipids provides clues on the mechanism of uptake, it is even more important to determine the distribution of intraliposomal drugs following uptake. ZnPC is a weak fluorophore; its intracellular localization can thus be investigated using confocal microscopy. In case of TTLs-VHH, where images were made at lower magnification to increase detection sensitivity at the expense of resolution, intracellular ZnPC was marginally visible (Fig. 4C). In contrast, cells incubated with TTLs+VHH exhibited relatively high levels of pan-cytoplasmic ZnPC (Fig. 4D). Co-incubation of cells with MTR (depicted in green) showed that ZnPC partly colocalized with mitochondria, but was also present at other intracellular locations. Given the lipophilicity of ZnPC (logP of ~8), the photosensitizer most likely localized to organelle membranes and/or proteins with hydrophobic binding pockets. In conclusion, the results in Fig. 3 and Fig. 4 indicate that TTLs+VHH represent an effective and selective drug delivery system for the intracellular delivery of lipophilic drugs such as ZnPC.

### 3.5 In vitro toxicity of anti-EGFR nanobodies and TTLs

The inherent toxicity of TTLs, its conjugated antibody, and the incorporated fluorophores...
and ZnPC were investigated in A431 and 3T3 cells. The data indicate that, up to a concentration of 200 μM, TTLs+VHH did not exert cytotoxicity in either A431 (Fig. 5A) or 3T3 2.2 cells (Fig. 5B). Neither TTLs nor Rho-PE are hence toxic to these cells. Since the delivery of ZnPC was substantially enhanced with the use of TTLs+VHH, it is important to determine whether high ZnPC payloads confer any dark toxicity. As shown in Fig. 5C, there was no notable dark toxicity induced by ZnPC after 48 h of incubation. Taken altogether, none of the liposomal constituents were toxic to cells.

3.6 In vitro PDT efficacy

Next, the toxicity of ZnPC-TTLs+VHH was tested in the context of PDT. A431 and 3T3 2.2 cells were incubated with the TTLs (125 μM) for 10 min and irradiated at a radiant exposure of 10 J/cm². Fig. 5D shows that PDT with ZnPC-TTLs without VHH induced minor levels of cell death in both A431 and 3T3 2.2 cells. However, PDT with ZnPC-TTLs+VHH induced 47% cell death in A431
cells, while 3T3 2.2 cells remained relatively unaffected. Reducing the VHH content of the liposomes to 1/5 of the original concentration also reduced the PDT-efficacy as both cell lines did not show reduced cytotoxicity in response to PDT. Thus, the TTLs+VHH were selective for EGFR-overexpressing cells, and the PDT-efficacy of the ZnPC-TTLs depended on the amount of VHH loaded onto the liposome surface. These results were corroborated by the ZnPC dosage data in Fig. 5E, which illustrate the dose-effect relationship between TTL+VHH concentration (i.e., the extent of uptake) and PDT efficacy (i.e., the extent of ZnPC-mediated ROS generation). Cell viability was determined after 10-min exposure to TTLs and a 120-min chase with fresh medium devoid of TTLs. TTLs+VHH were mildly toxic to 3T3 2.2 cells (triangles) in a concentration-independent manner. TTLs-VHH exerted a mild concentration-dependent toxicity on the A431 cells (squares), with a maximal induction of 28% cell death at the highest concentration (250 μM TTLs). This was in contrast to the PDT efficacy of TTLs+VHH in A431 cells (circles). PDT induced 43% cell death at a TTL lipid concentration of 15.6 μM. Next, we determined whether ZnPC was retained by the tumor cells and remained intact (i.e., not biotransformed to a non-functional metabolite) by increasing the drug-light interval. Cells were incubated with 125 μM TTLs for 10 min, and were irradiated at increasing drug-light intervals. A drug-light interval of 0.5 h increased the efficacy of TTLs+VHH — an effect that prevailed up to a drug-light interval of 4 h. At longer drug-light intervals, the efficacy of PDT decreased. This may have been caused by several factors: (a) ZnPC was excreted from the cells, (b) ZnPC underwent biotransformation (xenobiotic phase I or II metabolism), and/or (c) the ZnPC redistributed over time to organelles that trigger less extensive cell death signaling after PDT. The TTLs-VHH exhibited increased PDT efficacy at longer drug-light intervals, implying that these liposomes interacted with the tumor cells during the 10-min incubation period but were internalized at a slower rate compared to TTLs+VHH.

Figure 5. (A) Viability of A431 cells exposed to increasing concentrations of Rho-TTLs+VHH and Rho-TTLs-VHH. (B) Viability of 3T3 2.2 cells exposed to increasing concentrations of Rho-TTLs+VHH and Rho-TTLs-VHH. (C) Viability of A431 cells exposed to increasing concentrations of ZnPC-containing TTLs+VHH and TTLs-VHH in the absence of light irradiation (dark toxicity). All data represent the means ± standard deviations of N = 6. (D) Viability of A431 and 3T3 2.2 cells after incubation with either TTLs+VHH (dark grey bars), TTLs+1/5VHH (light grey bars), or TTLs-VHH (white bars) and subsequent PDT (10 J/cm2). Data represent the mean ± standard deviation of N = 3, statistics were performed using a student’s t-test. (E) Relative viability of A431 (black lines and symbols) and 3T3 2.2 cells (grey lines and symbols) after photosensitization with increasing concentrations of TTLs+VHH (squares) or TTLs-VHH (circles) and subsequent irradiation (10 J/cm2). Data represent the mean ± standard deviation of N = 6. (F) Relative viability of A431 cells after PDT with 125 μM TTLs+VHH (grey bars) or TTLs-VHH (white bars) as a function of the drug light interval.
3.7 TTL-cell interactions and time-based lipid distribution

To visualize TTL-cell interactions and internalization as well as time-dependent distribution of lipids in the cells, confocal laser scanning microscopy was performed on A431 and 3T3 2.2 cells after incubation with NBD-labeled TTLs+VHH or TTLs-VHH. Cells were co-stained with MTR (red) and DAPI (blue). Images were made after chase intervals of 0.5-, 4-, and 24 h. Fig. 6A shows that TTLs+VHH were quickly taken up by A431 cells, but the NBD fluorescence was substantially reduced after 4 and 24 h. Fig. 6B shows that TTLs-VHH were also taken up by A431 cells, albeit to a lesser degree relative to the TTLs+VHH. However, the TTLs-VHH were more stably retained by the cells. These results confirm that TTLs+VHH are quickly bound and internalized by A431 cells after which the liposomal lipids are distributed throughout the cells. The specificity of TTLs+VHH as determined in Fig. 5D and E were underscored by the images of Fig. 6C. 3T3 2.2 cells incubated with TTLs+VHH were negative for NBD fluorescence, indicating an absence of liposome uptake. The NBD fluorescence intensity in 3T3 2.2 cells was not influenced by the length of the chase interval.

4. Discussion

The goal of this study was to develop a method for the preparation of EGFR-targeted liposomes and to provide in vitro proof-of-concept that these liposomes can be utilized for the specific delivery of the photosensitizer ZnPC to EGFR-overexpressing tumor cells for application in PDT. The most important findings were that the circumstances for bacterial expression are best at 25 °C, thiol-mediated cleavage and NCL can be performed most optimally with CA, and that the VHH-CA can be effectively coupled to DSPE-PEGmal-containing liposomes to yield immunotargeted liposomes (TTLs+VHH). These TTLs+VHH exhibited superior uptake, ZnPC-delivery, and PDT-efficacy characteristics relative to TTLs-VHH. Moreover, the effects were specific for EGFR-overexpressing A431 cells and were not observed in EGFR-null 3T3 2.2 cells.

Nanobodies have previously been conjugated onto liposomes via random conjugation 21. Although these strategies were successfully adopted to yield immunoliposomes, the labeling efficiency was usually low, which was attributed to poor EPL performance. Therefore, this study aimed to optimize the EPL reaction adopting a relatively successful site-specific conjugation strategy as described in 25. The first step was to express, concentrate, and purify the nanobody, and ELPs were previously demonstrated to reversibly aggregate in response to increasing temperature and ionic strength and can thus be used to readily concentrate and purify the protein 26, 27. Thus, the nanobody-intein fusion protein was additionally coupled to an ELP-domain. Microbial expression could be maximized by growing cells in IPTG-supplemented medium for 20 h at 25 °C, after which the fusion protein could be easily purified and concentrated by utilizing ELP aggregation under high ionic strength.

Subsequently, EPL and NCL are necessary to further yield a functional nanobody. The NCL reaction is dependent on a reactive thioester 28. Thioesters, however, are also susceptible to hydrolysis and amidation and therefore need to be readily converted into the desired product. Circumvention of this intermediate step could also improve the EPL-efficiency. We therefore evaluated Cys analogs to perform the thiol-mediated cleavage (EPL) and the subsequent functionalization of the Cys (NCL). This reaction is known to depend on the C-terminal residue of the target protein as well as the reactivity of the thiol group 29. The more efficient EPL with CA compared to Cys can possibly be explained by the lower pKa (8.27 vs 8.7) 30. In contrast, BME has a much higher pKa (9.72) than CA but was remarkably faster. The intein-mediated cleavage by thioles therefore does not solely depend on a low pKa and the degree of formation of the corresponding thiolate at neutral pH (6-8), but also on other functional groups in the molecule that may possibly affect thiol-nucleophilicity. Furthermore, the positively charged amino group of e.g., CA might directly interact with the thiolate and/or prevent nucleophilic attack of the thioester. Another possibility is that the alcohol group of BME may act as a general base and aid in the partial deprotonation of the thiol group. Although the intein-mediated cleavage is most efficient in the presence of BME, CA is the preferred choice for
the introduction of a reactive thiol group, since both EPL and NCL can be performed with this compound. The labeling of proteins via Cys has drawn much attention since thiol groups are more nucleophilic than amines and are often present in proteins as disulfide bonds. An additional Cys allows for site-directed conjugation to maleimide groups, as a consequence of the thousand-fold selectivity for maleimide over amines at neutral pH\textsuperscript{31}. However, one major drawback is the non-native disulfide bond formation that is associated with protein misfolding. EPL with CA overcomes this problem by introducing the thiol group immediately after protein folding post-EPL.

In this study, a nanobody against the human EGFR receptor was utilized. EGFR is a tyrosine-kinase receptor that undergoes dimerization and autophosphorylation (activation) when EGF binds to its extracellular domain. It subsequently initiates intracellular signaling cascades via RAS, RAF, phosphatidyl inosital 3 requiring kinase (PI3K), and protein kinase C (PKC) to initiate proliferation and survival. EGFR is often overexpressed by tumors of varying origins\textsuperscript{32}, including tumor types that are eligible for, but typically respond poorly to, PDT, including bladder tumors\textsuperscript{33}, nasopharyngeal carcinoma\textsuperscript{34}, and bile duct cancers\textsuperscript{35}. EGFR has also been implicated in resistance to PDT, there-

Figure 6. Confocal laser scanning microscopy images of A431 (A and B) and 3T3 2.2 cells (C) after incubation with fluorescently (NBD)-labeled TTLs+VHH (A and C) or TTLs-VHH (B). NBD (liposomes) is shown in green, MTR (mitochondria) is shown in red, and DAPI (nuclei) is shown in blue. All images were made with a 63× oil immersion lens and digital zoom.
by signifying the importance of the development of a PDT regimen targeted at EGFR-overexpressing cells. Our data demonstrate the superior uptake kinetics and therapeutic efficacy of TTLs versus non-targeted liposomes.

The concept of immunotargeted liposomes has been extensively investigated over the years. For instance, a variety of immunotargeted liposomes have been prepared for the targeted delivery of doxorubicin to various malignancies, such as CD19-targeted liposomes for the treatment of B-cell lymphoma, EGFR-targeted liposomes for the treatment of breast cancer, glioblastoma, and ovarian carcinoma, and insulin-like growth factor 1 receptor-targeted liposomes for the treatment of pancreatic cancer. It is interesting to note that most studies in which immunotargeted liposomes were employed yielded favorable outcomes with respect to drug delivery and/or therapeutic efficacy in comparison to free therapeutic agents or non-targeted liposomes. Along the same lines, this study shows superior uptake kinetics and therapeutic efficacy of TTLs in comparison to non-targeted liposomes.

Most of these studies have used either conventional monoclonal antibodies or Fab fragments, and used non-site specific conjugation strategies to yield immunotargeted liposomes. As alluded to previously, the use of nanobodies holds several important advantages over conventional antibodies and Fab fragments. The size, stability, and cost of production are all superior in comparison to conventional ligands for immunotargeting. Moreover, the nanobody derived, purified, and functionalized in this study is interchangeable with a large variety of nanobodies directed at e.g. anti-HGF and anti-VEGFR2. Thus, the TTL formulation can be optimized using the described method for a plethora of different tumor types, depending on the known (over)expression of their surface receptors. Moreover, the TTL formulation can be used for the inclusion of different hydrophobic and hydrophilic drugs and, thus, the selective delivery of these drugs towards the target tissue. This represents a major advantage towards the use of TTLs for a diverse range of therapeutic applications.

5. Conclusions

This study demonstrated an efficient and feasible method to obtain purified nanobodies that can be conjugated to photosensitizer-containing liposomes that can be applied for PDT with high specificity. It was demonstrated that nanobodies can be efficiently functionalized by EPL using CA, and that the functionalized nanobodies could be site-specifically conjugated to DSPE-PEG-mal-containing liposomes (TTLs). These TTLs were readily taken up by EGFR-overexpressing tumor cells but not by EGFR-null cells, were minimally toxic in absence of light irradiation, and showed high PDT efficacy in comparison to non-functionalized liposomes. Our PDT strategy warrants further in vivo validation, which is currently underway. Moreover, the nanobodies used in this study are interchangeable, as are the intraliposomal drugs. As such, the methods described in this study may easily be adapted to suit the needs for a broad range of applications in which targeted drug delivery is desired.

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