Chapter 23

In Vitro Assessment of Binding Affinity, Selectivity, Uptake, Intracellular Degradation, and Toxicity of Nanobody-Photosensitizer Conjugates

Irati Beltrán Hernández, Timo W. M. De Groof, Raimond Heukers, and Sabrina Oliveira

Abstract

Photosensitizers have recently been conjugated to nanobodies for targeted photodynamic therapy (PDT) to selectively kill cancer cells. The success of this approach relies on nanobody-photosensitizer conjugates that bind specifically to their targets with very high affinities (k_D in low nM range). Subsequently, upon illumination, these conjugates are very toxic and selective to cells overexpressing the target of interest (EC_{50} in low nM range). In this chapter, protocols are described to determine the binding affinity of the nanobody-photosensitizer conjugates and assess the toxicity and selectivity of the conjugates when performing in vitro PDT studies. In addition, and because the efficacy of PDT also depends on the (subcellular) localization of the conjugates at the time of illumination, assays are described to investigate the uptake and the intracellular degradation of the nanobody-photosensitizer conjugates.

Key words Nanobody-photosensitizer conjugate, Binding affinity, Selective toxicity, Co-cultures, Uptake, Intracellular degradation

1 Introduction

Photosensitizers have recently been conjugated to nanobodies for targeted photodynamic therapy (PDT) to selectively kill cancer cells [1–8]. Nanobodies are the smallest naturally occurring antigen-binding domain, derived from heavy-chain antibodies found in Camelids [9], and have been employed in many different applications [10–14]. One of the strongest points of nanobody-targeted PDT is the selectivity and specificity for killing tumor cells. The photosensitizer is selectively delivered to the cells which overexpress the target of interest on the cell membrane, such as the epidermal growth factor receptor (EGFR).

Irati Beltrán Hernández and Timo W. M. De Groof contributed equally to this work

Mans Broekgaarden et al. (eds.), *Photodynamic Therapy: Methods and Protocols*, Methods in Molecular Biology, vol. 2451, https://doi.org/10.1007/978-1-0716-2099-1_23,

Nanobody-photosensitizer conjugates bind differently to cells which have different expression levels of the target and, most importantly, nanobody-targeted PDT can selectively kill those cells with the highest target expression, while negative or low-expressing cells remain unaffected [1, 2].

The success of the nanobody-targeted PDT approach relies on nanobody-photosensitizer conjugates that bind specifically to their targets with very high affinities (k_D in low nM range). Although nanobodies are known to bind with very high affinities to their targets, a nanobody-photosensitizer conjugate may have its binding properties affected by the conjugation of the photosensitizer. It is therefore essential to determine the binding affinity of the conjugate, as low affinity will likely result in poor accumulation at the tumor site in vivo [15, 16]. Here, we describe a stepwise protocol to determine the binding affinity of nanobody-photosensitizer conjugates, performing binding assays on cells. After this, we describe an assay to determine the toxicity of the nanobody-photosensitizer conjugates on cells, when the photosensitizer is activated by the appropriate light. Here, a method in a 96-well plate format is described, allowing for testing a wide range of concentrations to obtain EC50 values (usually in the low nM range). Furthermore, this approach relies on conjugates that are very toxic to cells overexpressing the target of interest while leaving cells with low target expression unaffected. It is very important to assess this selectivity, as some tumor markers are also present in normal tissues surrounding tumors, though at a lower degree. For this, a method is here described involving co-cultures of cells with different expression levels.

In general, the efficacy of PDT is also partly determined by the (subcellular) localization of the photosensitizers upon illumination [17–22]. Because the reactive oxygen species generated by the photosensitizer travel only short distances, PDT will only generate cell damage near the site where they are generated [18, 23]. The localization of photosensitizer generally depends on its chemical properties, such as molecular weight, hydrophobicity, and charge [24, 25]. Some photosensitizers show preferred intracellular localization in the plasma membrane, while others end up in intracellular organelles like mitochondria, endoplasmic reticulum, Golgi, and lysosomes [22]. However, in this approach of nanobody-targeted PDT, the nanobody is the main driver for the distribution of the photosensitizer. For example, nanobody-photosensitizer conjugates could be taken up by the target cells via endocytosis [1, 26]. The different intracellular routes that the receptors can take could potentially be tuned and exploited to maximize the efficacy of the PDT. Processes like receptor activation, clustering, or passive uptake cause internalization of the receptors into early endosomes [26, 27]. Internalized receptors subsequently recycle back to the membrane via different types of recycling vesicles or

traffic toward protein degradation, which involves late endosomes, multiple vesicular bodies, and lysosomes. Alternatively, proteins can be degraded via the ubiquitin-proteasome system (UPS) [27]. The different localization and chemical nature (pH, reducing environment) affect the extent of the damage caused by light activation and hence the efficacy of PDT [28–30]. Because of this, determining the intracellular fate of nanobody-photosensitizer conjugates is a relevant part of their in vitro characterization. This chapter describes examples for determining the extracellular and intracellular fraction of nanobody-photosensitizer conjugates. Next, a method for determining the ratio of internalized/total nanobody-photosensitizer conjugate and an approach to study the degradation of the conjugates are described.

Altogether, this chapter describes essential protocols to characterize and investigate nanobody-photosensitizer conjugates in the in vitro setting.

2 Materials

2.1 General Materials

1. Cells with and without expression of the target of interest.
2. Cell culture medium, such as Dulbecco's modified Eagle medium (DMEM) + 10% fetal bovine serum (*see* **Note 1**).
3. Milli-Q (18 M∧-cm).
4. 1x PBS: 138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 in Milli-Q; adjusted to pH 7.4 with HCl.
5. Nanobody-photosensitizer conjugates in PBS.
6. Odyssey Infrared Scanner (LI-COR) or a similar device for detection of near-infrared wavelengths.

2.2 Binding Assay on Cells for Affinity Determination

1. Binding medium: 1% BSA and 25 mM HEPES in Dulbecco's modified Eagle medium (DMEM) without phenol red. Adjusted to pH 7.2. Stored at 4 °C.
2. GraphPad Prism or similar standard analysis software.

2.3 In Vitro Nanobody-Targeted PDT and Toxicity Assessment

1. PDT medium: Dulbecco's modified Eagle medium (DMEM) without phenol red, supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin.
2. Light source applicable to a 96-well plate format, for instance a device consisting of 96 LED lamps (670 ± 10 nM, 1 LED per well) [31, 32].
3. Laser measurement sensor (Ophir Optronics LTD), or similar optometer able to measure light intensity at 690 nm.
4. Alamar Blue reagent (BIO-RAD).

5. FluoStar Optima fluorescent plate reader (BMG Labtech GmbH) or similar plate reader capable of exciting at 550 nm and detecting emission at 590 nm.
6. GraphPad Prism or similar standard analysis software.

2.4 Live/Dead Cell Assay with Mono- and Co-Cultures

1. Propidium iodide (Invitrogen).
2. Calcein AM (Invitrogen).
3. EVOS Microscope (Advanced Microscopy Group, AMG, Thermo Fischer Scientific) equipped with 10× objective, and GFP and RFP (or Texas Red) LED-based fluorescence light cubes, or other similar microscopes.

2.5 Internalization Assay

1. Acid wash buffer. Either:
   (a) 0.2 M Glycine buffer, 0.15 M NaCl pH 3.0 [33]
   (b) 0.05 M Glycine buffer, 0.15 M NaCl pH 3.0 [34]
   (c) 0.2 M Acetic acid, 0.5 M NaCl pH 2.5 [35, 36]
   (d) Acid stripping buffer: DMEM supplemented with 0.2% bovine serum albumin (BSA) and with the pH adjusted to 3.5 using HCl [37]
   (e) DMEM, 10 mM HEPES, 0.2% BSA pH 2.5 [38]
2. A detector for quantification of the label. This can either be a fluorescence scanner or a radioactive counter. ^{125}I-labeled nanobodies can be quantified using a PerkinElmer Precisely 1470 automatic gamma counter [26]. In case of the PS IRDye700DX, the PS can be quantified using an Odyssey scanner (Li-COR) [1].
3. ELISA materials: Antibodies directed against the nanobodies or tags (e.g., anti-VHH, anti-Myc or anti-His). Peroxidase-conjugated secondary antibodies and detectable colorimetric (o-phenylenediamine (OPD), tetramethylbenzidine TMB), fluorescent, or chemiluminescent substrate, combined with the appropriate detector.
4. TCA precipitation: 20% (w/v) Trichloroacetic acid (TCA) in Milli-Q.

2.6 Intracellular Degradation Assay

1. Size separation: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffers and equipment.
2. SDS-PAGE loading buffer without bromophenol blue. 200 mM Tris–HCl, pH 6.8, 40% glycerol, 400 mM DTT, 8% w/v SDS.

3 Methods

Keep the nanobody-photosensitizer conjugates at 4 °C or −20 °C and protected from light.

3.1 Binding Assay on Cells for Affinity Determination

1. Seed cells in a 96-well plate using 100 μL per well, in such a way that cells reach around 85–90% confluency on the day of the assay (*see* **Note 2**). Perform the assay in triplicates, meaning cells should be seeded in ten by three wells per each conjugate to be tested, leaving the outer rim without cells, to avoid artifacts due to the faster evaporation of the medium in those wells.
2. On the day of the assay, let the cells adjust to room temperature for 5 min. Subsequently, bring the cells to 4 °C and let settle for another 5 min (*see* **Note 3**).
3. Prepare a dilution of nanobody-photosensitizer conjugate in binding medium in a final concentration of 100 nM (*see* **Note 4**).
4. Remove culture medium from the wells and wash cells once with cold binding medium.
5. Add a concentration range of conjugate starting with the highest concentration and diluting 1:2 with binding medium in each consecutive column of wells. The final volume in each well should be 100 μL.
6. Incubate the cells with the conjugate for 2 h at 4 °C, with gentle agitation. Keep the well plate protected from light.
7. Wash cells twice with cold binding medium and remove any remaining bubbles.
8. Scan the well plate to detect the bound nanobody-photosensitizer conjugate via the fluorescence of the photosensitizer. Scan at 700 nm when using the photosensitizer IRDye-700DX.
9. Plot the nanobody-photosensitizer concentration on the x-axis and the fluorescence intensity values on the y-axis (Fig. 1a), after subtracting background fluorescence corresponding to wells with cells and medium only. From the resulting saturation curve, calculate the concentration at which half of the maximum fluorescence is observed, which is known as the apparent affinity of the nanobody-photosensitizer conjugate (*see* **Note 5**).

3.2 In Vitro Nanobody-Targeted PDT and Toxicity Assessment

When setting up and performing the illumination step for PDT, wear laser safety glasses and ensure that others take precautions as well.

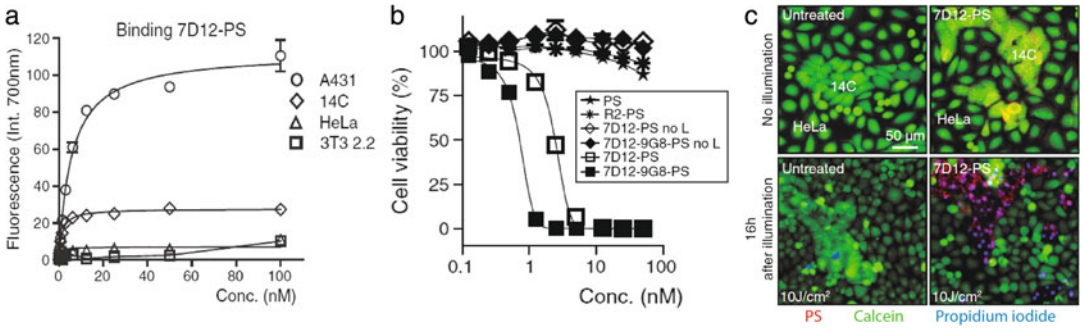

Fig. 1 Specific binding and selective toxicity of nanobody-photosensitizer conjugates. (**a**) Nanobody-photosensitizer conjugates were incubated with cells in a concentration range and bound conjugate was detected. Conjugates bind to cell lines with different levels of EGFR (A431 > 14C > Hela), but not to negative cells (3 T3 2.2). (**b**) Cell viability of 14C cells after nanobody-targeted PDT expressed as a percentage relative to untreated cells. The photosensitizer (PS) alone or light alone does not have an effect on cell viability; only the combination of conjugate (7D12-PS or 7D12-9G8-PS) with light results in cell toxicity. (**c**) Fluorescent microscopy of a co-culture of Hela and 14C cells when no light is applied and after nanobody-targeted PDT. Propidium iodide staining (depicted in blue) can only be detected on 14C cells after treatment, while Hela cells remain alive as depicted by the calcein staining (in green). The 7D12-PS conjugate (shown in red) can be imaged as well [1]

1. Seed cells in a 96-well plate using 100 µL per well, in such a way that cells reach around 70–75% confluency on the day of the assay (*see* **Note 2**). Perform the assay in triplicates, meaning cells should be seeded in ten by three wells per each conjugate to be tested. Do not seed cells in the outer wells in order to avoid artifacts due to the faster evaporation of the medium in those wells.
2. On the day of the assay, prepare three dilutions of the nanobody-photosensitizer conjugate in PDT medium (e.g., 50 nM, 5 nM, and 0.5 nM).
3. Remove the cell culture medium from the wells and wash once with warm PDT medium using 100 µL per well.
4. Add the nanobody-photosensitizer conjugate, starting at 50 nM and diluting 1:2 with PDT medium in the two consecutive columns of wells (for 25 and 12.5 nM). Do the same starting with 5 nM and 0.5 nM (*see* **Note 6**). The final volume in each well should be 100 µL. Use the last column of wells with cells as control wells, which will only contain 100 µL of PDT medium, and thus no conjugate (*see* **Note 7**).
5. Incubate the well plate at 37 °C (5% CO_2) for 30 min; this step is also referred to as pulse (*see* **Note 8**).
6. During the incubation time, set up the light-emitting device. Using the optometer and covering the device with black paper, or a black plastic box, measure the intensity of the emitted light at the height of the well plate. Adjust this intensity to the desired value (e.g., 4 mW/cm^2).

7. After the pulse, wash cells twice with PDT medium and add 100 μL of PDT medium to all the wells (including outer wells).
8. Immediately after the washes and just before illumination, use the Odyssey infrared scanner to detect the fluorescence corresponding to the nanobody-photosensitizer conjugate associated with cells (bound and/or internalized) (*see* **Note 9**).
9. Always cover the bottom of the control wells with black paper, where no nanobody-photosensitizer conjugate was added, so that these wells do not receive light. Illuminate with the desired fluence and fluence rate (e.g., a fluence rate of 4 mW/cm^2 for 42 min, for a total dose of 10 J/cm^2). Cover the plate with black paper during the illumination time, for safety reasons.
10. Place the well plate back in the 37 °C incubator after illumination.
11. After overnight or 24 h, bring Alamar Blue reagent to room temperature and add 10 μL to each well. Mix this with the 100 μL of medium already present in the wells (*see* **Note 10**). Make sure to add Alamar Blue reagent to some outer wells (without cells) to set the background fluorescence signal.
12. Return well plate to 37 °C and incubate for 1.5 h, or until a purple to pink color develops in the control wells (*see* **Note 11**).
13. Measure the fluorescence intensity with a well plate reader. Take as background the values from wells only containing PDT medium and Alamar Blue. Set the 100% viability from the wells that did not receive light and were not treated with nanobody-photosensitizer conjugate (control wells). Express cell viability as a percentage relative to these control wells (Fig. 1b). The concentration at which 50% of the cells are killed is the EC_{50} and can be determined using GraphPad.

3.3 Live/Dead Cell Assays with Mono- and Co-Cultures

1. Seed cells in a 96-well plate using 100 μL per well, in such a way that cells reach around 70–75% confluency on the day of the assay. When using co-cultures, seed a mixture of cells consisting of 50% of each cell line (*see* **Note 12**).
2. Perform nanobody-targeted PDT on the 96-well plate (as described above) using the nanobody-photosensitizer conjugate concentrations of interest. For instance, 25 nM nanobody-photosensitizer is enough to result in 100% toxicity to the cells.
3. Incubate the well plate at 37 °C for the desired time. Live/dead assessment can be performed as early as right after PDT or after overnight incubation. Necrotic cells are already detectable early after nanobody-targeted PDT.

4. Dilute propidium iodide and calcein AM in PBS to a concentration of 1 μg/mL and 0.5 μg/mL, respectively (*see* **Note 13**). Remove the culture medium from the wells and add 100 μL of solution per well. Incubate the well plate at 37 °C for 5 min.
5. Image the cells with the EVOS Microscope using the GFP channel for calcein AM and the RFP channel for propidium iodide. Dead cells will appear red, while live cells will be stained with green (Fig. 1c). Phase-contrast images can also be taken with the EVOS Microscope and overlays are generated.

3.4 Internalization Assay

This assay is employed to investigate the uptake of nanobody-photosensitizer conjugates, but is also applicable to conjugates of nanobody with radioisotopes or fluorophores.

For determining the ratio of internal vs. total/external nanobody-photosensitizer conjugates, it is important to be able to accurately remove or isolate the extracellular nanobody-photosensitizer fraction. Because binding of the nanobody is often mediated by charge interactions, extracellular binding nanobody can be removed using an acid wash. However, to validate whether the acid wash is successful, the binding of the nanobody-photosensitizer to the target after acid wash at 4 °C (*see* **Note 14**) needs to be assessed. This is of importance because some nanobodies are still able to bind their targets at acidic pH.

3.4.1 Determination of the Binding Equilibrium of the Nanobody Over Time

To determine the effectivity of the acid wash, the binding equilibrium of the nanobody needs to be determined first by testing the binding of a saturating concentration over time (*see* **Note 15**).

1. Seed cells in a 96-well plate to obtain confluency the next day.
2. Take the plate on ice (or 4 °C) the next day and aspirate the growth medium of the latest time point. Add 100 μL of the saturating concentration of nanobody-photosensitizer, diluted in cold blocking buffer, to the well (*see* **Note 16**).
3. Do the same for the next time points. After incubation of the conjugates at different time points, aspirate all wells and carefully wash all wells 2–3 times with 150 μL of cold PBS on all wells.
4. The fluorescence intensity of the cell-bound nanobody-photosensitizer conjugates can be quantified using a preferred method (*see* **Note 17**).

3.4.2 Optimization of the Acid Wash

A critical part of this protocol is the acid wash, used to remove bound nanobody-photosensitizer conjugates from the surface of the cells, to accurately investigate the intracellular fraction. Therefore, it is important to optimize this wash, so that it is compatible with the conjugate being investigated.

Careful consideration is needed when choosing the appropriate acid wash buffer. Different types of acid wash can be used and are summed up in the material section. To assess the effectivity of the acid wash buffer, a saturating concentration of nanobody-photosensitizer is added to cells and incubated until binding equilibrium is reached.

1. Seed cells in a 48-well plate to obtain confluency the next day. In case of cells that detach easily, cells can be seeded 2 days before the assay.
2. Take the plate on ice (or 4 °C) the next day and aspirate the growth medium. Add 100 μL of a saturating concentration of nanobody-photosensitizer, diluted in cold blocking buffer, and incubate long enough to reach binding equilibrium (as described in Subheading 3.5.1, *see* **Note 18**).
3. Remove the cold medium and add 150 μL of acid wash buffer (*see* **Note 19**) for 10 min on ice. Aspirate or collect the acid wash buffer and wash again with 150 μL of acid wash buffer (*see* **Note 20**).
4. Aspirate or collect the acid wash buffer from all wells.
5. Wash the wells 3× with 200 μL ice-cold washing buffer while keeping the plate on ice.
6. Scan the plate using a fluorescence scanner to determine the amount of residual nanobody-photosensitizer conjugates after acid wash. Alternatively, in case the acid wash is tested using radiolabeled nanobodies, lyse the cells in 150 μL RIPA buffer for 10 min at room temperature and quantify the residual nanobodies using a radioactivity counter.

3.4.3 Determining Internalized Nanobody-Photosensitizer Fraction Using Fluorescence

Using an optimized acid wash protocol allows a careful distinction between total, surface-bound, and internalized nanobody-photosensitizer conjugates. This can be assessed by different methods. For example, the traceability of the nanobody-photosensitizer conjugates can be exploited by detecting them by their fluorescent properties. However, uptake of conjugates can also be quantified by using radiolabeled nanobodies or via ELISA.

1. Seed cells, with (and without) the receptor of interest, in two 48- or 96-well plates to obtain confluency on the day of the assay (*see* **Note 21**). One plate will be tested at 37 °C (warm plate where uptake takes place) and one at 4 °C (cold plate with only surface binding).
2. Next day, take the cold plate on ice and dilute the nanobodies at saturation concentration in prewarmed (37 °C) or cold (4 °C) binding medium. Alternatively, the cells can be preincubated with the nanobody-photosensitizer solution at 4 °C,

until binding equilibrium is reached, after which the plates are transferred to 37 °C for the desired time periods.

3. Aspirate the medium from the wells of the last time point of both the warm and cold plates and add 100 or 150 μL (for 96- or 48-well plate, respectively) of the nanobody in the binding medium. Put the plate back in the incubator or on ice.
4. Do the same for the next time points. After incubation of nanobodies at different time points, quickly aspirate all wells and put 150–200 μL of ice-cold PBS on all wells.
5. Wash wells again with cold PBS and apply the acid wash as optimized under Subheading 3.4.2. The amount of label in this acid wash can be regarded as bound, but not internalized nanobody-photosensitizer. The acid wash should have removed all nanobody-photosensitizer from the cells that have been kept at 4 °C (*see* **Note 22**).
6. Scan the plate after this acid wash in order to quantify the internalized fraction of photosensitizer. Alternatively, lyse the cells in SDS-PAGE loading buffer and quantify the amount of label on a fluorescence scanner after size separation by SDS-PAGE.

3.4.4 Determining Internalized Nanobody-Photosensitizer Fraction Using ELISA

In case the internalization on nanobodies cannot be measured directly via their label (photosensitizer, fluorophore, radioactivity, etc.), one might consider to determine the nanobody uptake by ELISA. Nanobodies can be detected with anti-VHH antibodies or via the use of specific peptide tags (Myc, FLAG, His).

1. The first steps in this protocol are similar to the first five steps described under Subheading 3.4.3.
2. Fix the cells for 10 min with 100 μL (96-well plate) 4% paraformaldehyde in PBS.
3. Wash the cells with PBS and block the wells with 150 μL (96-well plate) blocking buffer for 30 min at room temperature.
4. Remove the blocking buffer and add the appropriate dilution of the nanobody in the blocking buffer to detect the tag. Incubate the primary antibody solution long enough to allow binding equilibrium at room temperature.
5. After reaching binding equilibrium, wash the wells 2–3x with PBS and add a secondary antibody-HRP dilution to the wells. If extra incubation steps are needed to detect the receptor of interest, repeat this step.
6. Wash the cells 3x with PBS and add OPD/TMB to the wells.
7. Stop the reaction with 1 M H_2SO_4 in time to allow maximum window.

8. Quantify the fraction of internalized nanobodies (*see* **Note 23**) by spectrometry at a wavelength of 450 nm or 490 nm depending on the used substrate.

3.5 Assessment of Intracellular Degradation of Nanobody-Photosensitizer Conjugates

Upon uptake by the targeted cells, nanobody-photosensitizer conjugates can be recycled back to the membrane and secreted back into the medium or the conjugates can be degraded intracellularly by the proteasome system or the lysosomes. The free photosensitizer that is formed as a consequence of nanobody-photosensitizer degradation can either accumulate inside the cells or diffuse out of the cells into the medium. Free photosensitizer (or other label/fluorophore) and protein-bound photosensitizer (or label/fluorophore) can be quantified upon protein precipitation by means of TCA precipitation or by size separation by SDS-PAGE. The following protocol describes how to determine the fraction of degraded nanobody-photosensitizer conjugates by using SDS-PAGE and fluorescence imager.

1. Seed cells in a 48-well plate to obtain confluency the next day.
2. The next day, if desired, cells could be preincubated with inhibitors (*see* **Note 24**). Subsequently, take the plate on ice (or 4 °C) the next day and aspirate the growth medium. Add 100 μL of a saturating concentration of nanobody-photosensitizer, diluted in cold blocking buffer, and incubate long enough to reach the binding equilibrium.
3. Transfer the cells to 37 °C for the desired time periods.
4. For photosensitizer molecules that diffuse out of cell upon degradation of the conjugates, collect the medium and determine the fraction of degraded nanobody-photosensitizer conjugates by TCA precipitation of all proteins. For photosensitizer molecules that cannot diffuse out of cells (e.g., IRDye700DX), make cell lysates in sample buffer without bromophenol blue (*see* **Note 25**).
5. Determine the fraction of nanobody-photosensitizer conjugates and free photosensitizer upon size separation of the cell lysates by SDS-PAGE and quantification on a fluorescence scanner. An example of such a quantification is shown in Fig. 2.

4 Notes

1. Alternatives to DMEM medium as growth medium can be used.
2. To determine the affinity of the nanobody-photosensitizer conjugates, the use of a cell line with medium to high expression levels of the target is advised. This assay should also be performed with a negative cell line to demonstrate the

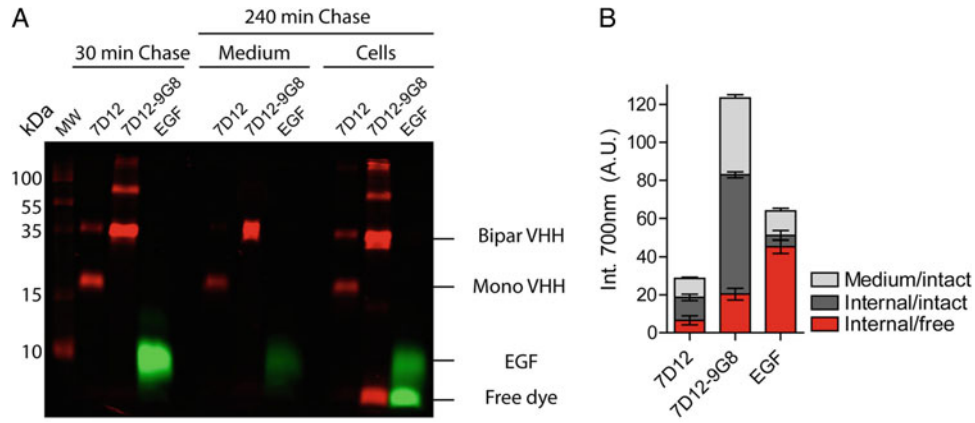

Fig. 2 Quantification of nanobody-photosensitizer and free photosensitizer upon a pulse-chase experiment in cells. (**a**) Cells treated with either IRDye680-labeled monovalent nanobody (7D12) or bivalent nanobody (7D12-9G8) or IRDye800-EGF were lysed and size-separated by SDS-PAGE. Ligand-bound fluorophore is visible at their height, while free fluorophore is present at the running front of the gel. (**b**) Ligand-bound and free fluorophore was visualized and quantified using an Odyssey imager (Li-COR) [1]

specificity of the conjugate. Cell lines with different target expression levels are also of use to further characterize the conjugate.

3. Ideally, the assay should be performed in a refrigerated room at 4 °C. Alternatively, perform the assay while keeping the plate on ice.
4. In general, we expect an apparent affinity in the nanomolar range; thus starting the assay with 100 nM will give a good saturation curve. However, if the outcome is uncertain, higher starting concentrations can be used, e.g., 1 μM.
5. The apparent affinity (K_D) can be determined using a one-site-specific binding, nonlinear regression protocol with GraphPad Prism, or similar standard analysis program. The apparent affinity should remain in the same range as the apparent affinity of the unconjugated nanobody in order to conclude that the conjugation of the photosensitizer to the nanobody does not affect its binding capacity. When a drop in affinity is detected (greater than one order of magnitude), it might be due to the presence of lysine in the complementary-determining regions or CDRs of the nanobody. Moreover, too much conjugated photosensitizer may also negatively affect the binding of the nanobody. The regression protocol will also determine the Bmax, which refers to the maximum density of receptors on the cells and gives an idea of the epitope availability. Thus, cell lines with different expression levels will result in graphs with different Bmax, where a direct correlation is observed.

6. By adding a concentration range starting from three stock dilutions, a wider range of concentrations can be covered (0.125–50 nM), than if only with 1:2 dilutions steps.
7. When assessing a nanobody-photosensitizer conjugate for the first time, a control consisting of conjugate but no light should be included. The light alone (10 J/cm^2) has already been shown to have no effect on the viability of several cell lines. However, it is recommended to include this control when performing the assay the first time.
8. This short incubation period, referred to as "pulse," is intended to simulate the short time that nanobody-photosensitizer conjugates have in vivo to accumulate at the tumor site due to their short half-life.
9. Since this incubation takes place at 37 °C, internalization of the nanobody can occur. If using a monovalent nanobody, the majority will likely not be internalized in the short incubation of 30 min. However, bivalent or biparatopic nanobodies can induce dimerization of the receptor and consequent internalization of the nanobody together with the receptor. The Odyssey scanner does not allow to differentiate fluorescence corresponding to bound nanobody from the internalized nanobody; thus the obtained fluorescence intensity values correspond to both nanobody fractions.
10. The active ingredient of Alamar Blue reagent is a cell-permeable blue compound which is nonfluorescent. When entering live cells, this compound is reduced and becomes red in color and highly fluorescent. Therefore, the fluorescence and color of the media surrounding viable cells will increase.
11. The incubation time with Alamar Blue will ultimately depend on the cell line and treatment. For some cases, 1 h at 37 °C is enough for the purple to pink color to develop in the control wells, while it might take up to 4 h in other cases.
12. Co-cultures of two cell lines can be used to assess the selectivity of nanobody-targeted PDT. For instance, use one cell line with low target expression and another one with medium to high expression. The cell lines can be differentiated based on the morphology and the amount of bound PS (Cy5 channel with EVOS microscope), or with a control assay with a fluorescent ligand (e.g., EGF-Alexa555 [1]).
13. Propidium iodide is membrane impermeant and will only gain access to the inside of the cell when the membrane is not intact. The fluorescence of propidium iodide increases when it intercalates with nucleic acids. On the other hand, calcein AM is cell permeant and becomes fluorescent in live cells because of their intracellular esterase activity. Therefore, propidium iodide is

used to identify necrotic cells with membrane damage, while calcein stains live cells.

14. The acid wash needs to be tested below 16 °C and preferably at 4 °C to prevent trafficking of the receptor of interest.
15. The time to reach binding equilibrium differs among nanobodies. If the binding equilibrium is not known, it is recommended to use 1 h at least as the latest time point.
16. When plating the cells, use saturating concentration of nanobody-photosensitizer (10x K_D). For determining the binding affinity, see the above Subheading 3.1. If possible, an irrelevant nanobody is also taken along as extra control.
17. If the nanobodies are conjugated to the photosensitizer or to a radioligand, binding can be quantified using a scanner. Other methods to detect nanobodies can include ELISA (see below).
18. One time point can be enough to check the acid wash but it is recommended to check different time points of incubation of the nanobody with the cells when assessing the effectivity of the acid wash buffer.
19. Different acid wash buffers have been used in literature and have been summarized in the material section. If a certain buffer does not work or has a toxic effect on the cells used in the assay, it is recommended to try a different acid wash buffer.
20. Preferably, wells containing cells with and without the receptor of interest are also incubated with the nanobody-photosensitizer but no acid wash is performed to assess the total fraction.
21. For both plates, cells expressing the receptor of interest need to be plated that will be incubated with saturating concentrations of nanobodies for different time points.
22. Also keep some wells where the nanobody has been incubated for the longest time period that are not incubated with the acid wash buffer. This ensures to determine the total fraction both on the cold and warm plates.
23. In case cells were preincubated with nanobodies at 4 °C, the ELISA signal in the cold plate can be regarded as the total signal in your assay. The internalized fraction is determined by the ratio of the signal in warm plate (internalized) and cold plate (total, no internalization).
24. If desired, inhibit lysosomal degradation by treatment with chloroquine (50 μM) or increasing the pH with NH_4Cl (20 mM) or inhibit proteasomal degradation with MG132.
25. Some photosensitizers of fluorophore molecules might show a spectral overlap with bromophenol blue in Laemmli sample buffer (such as IRDye700DX). In SDS-PAGE, free PS will be

run together with the bromophenol blue at the front of the gel. In case the free PS needs to be quantified, it is advisable to omit the bromophenol blue from these samples.

Acknowledgments

The authors received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement No. 677582) and the Netherlands Organization for Scientific Research (NWO: Vici grant 016.140.657).

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