Chapter 22

Conjugation of IRDye Photosensitizers or Fluorophores to Nanobodies

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Abstract

Fluorophores have been conjugated to nanobodies for approximately a decade, for several applications in molecular biology. More recently, photosensitizers have been conjugated to nanobodies for targeted photodynamic therapy (PDT). The most common chemistry is the random conjugation in which commercial fluorophores or photosensitizers contain a N-hydroxysuccinimide ester (NHS ester) group that reacts specifically and efficiently with lysines in the amino acid sequence of the nanobody and with the N-terminal amino groups to form a stable amide bond. Alternatively, maleimide-containing fluorophores or photosensitizers can be used for conjugation to thiols, in a site-directed manner through a cysteine incorporated at the C-terminal of the nanobody. This chapter addresses both conjugation strategies, providing details on the reaction conditions, purification, and characterization of the conjugates obtained.

Key words Nanobody, Photosensitizer, Fluorophore, Random conjugation, Site-directed conjugation

1 Introduction

Fluorophores have been conjugated to nanobodies for approximately a decade, for several applications in molecular biology, such as to investigate the internalization of the epidermal growth factor receptor, EGFR [1, 2]. Near-infrared fluorophores have been conjugated to nanobodies and particularly employed for preclinical optical molecular imaging of tumors. Our laboratory has developed nanobodies for optical imaging of EGFR [3, 4], HER2 [5, 6], and CAIX [6, 7] in human tumor xenografts grown in mice. More recently, our group has conjugated photosensitizers to nanobodies for targeted photodynamic therapy (PDT) [8–11]. In general, two main strategies can be used for such conjugations: (a) a random conjugation employing a fluorophore or photosensitizes with a N-hydroxysuccinimide (NHS) ester that reacts with lysine amino acids in the nanobody sequence and the N-terminal amino group and (b) a site-directed conjugation in which the fluorophore or

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_22,

photosensitizer contains a maleimide (Mal) group that can react with thiols, as present in cysteines, preferably added at the C-terminal of the amino acid sequence.

In the random conjugation, lysine residues in the amino acid sequence are acylated with fluorophore or photosensitizer molecules, forming a new amide bond. Since this reaction occurs at random, the degree of conjugation (DOC) depends on the number and accessibility of the lysines, and thus may vary for each different nanobody sequence. Importantly, forcing the reaction for maximizing the DOC should be done with caution, as this could compromise the binding properties of the conjugate to the target of interest.

The second strategy is a Michael addition of a thiol, present on a cysteine amino acid, to a maleimide linked to the fluorophore of photosensitizer, forming a thioether bond. Theoretically, it is possible to force the reaction for maximizing the DOC without compromising the binding properties of the conjugate to the target of interest. Such optimization can be done through variations in pH, incubation time, temperature, and nanobody-to-photosensitizer or -fluorophore molar ratio. Nevertheless, these variations should always be followed by determination of binding affinities to confirm that the nanobody remains functional post-conjugation.

This chapter addresses both conjugation strategies (Fig. 1), providing details on the reaction conditions, purification, and characterization of the conjugates obtained. This protocol focuses on the fluorophores and photosensitizers commercially available, IRDye 700DX NHS or IRDye 700DX Mal and IRDye 800CW NHS or IRDye 800CW Mal, but can serve as a reference even when other photosensitizers or fluorophores are to be conjugated to nanobodies or other targeting moieties.

2 Materials

2.1 General

1. 1x PBS: 138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 in Milli-Q; adjust to pH 7.4 with HCl.
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. Nanobody dissolved in 1x PBS.
4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffers and equipment.
5. IRDye 700DX NHS or IRDye 700DX Mal and IRDye 800CW NHS or IRDye 800CW Mal (LI-COR Biosciences) dissolved in anhydrous DMSO.
6. Zeba spin desalting columns (2 mL and 5 mL).

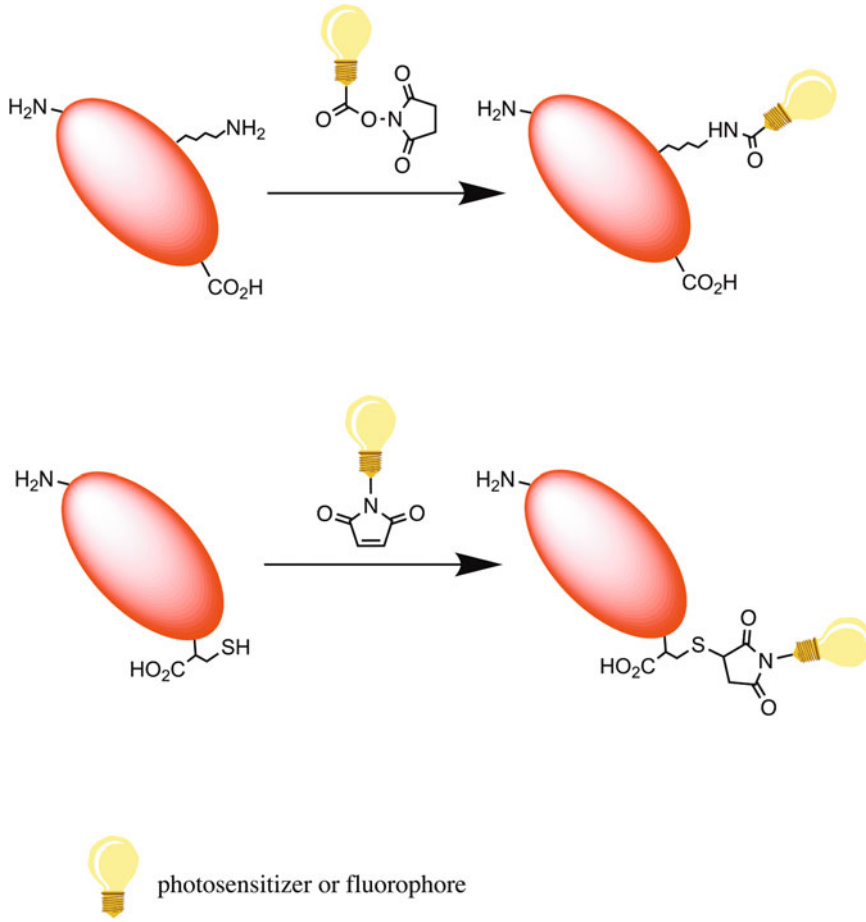

Fig. 1 Schematic representation of the random conjugation (top) and the site-directed conjugation (bottom)

7. Amicon ultracentrifugal filter (0.5 mL and 4 mL).
8. NanoDrop Spectrophotometer (Thermo Fisher Scientific).
9. Odyssey infrared scanner (LI-COR).

2.2 Site-Directed Conjugation

1. Tris buffer: 50 mM Tris; adjust to pH 8.5.
2. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP).
3. PBS-EDTA: PBS pH 7.4 supplemented with 0.4 mM EDTA.
4. Sodium phosphate buffer (50 mM) with 500 mM NaCl and 1 mM EDTA, adjust to pH 6.9.

3 Methods

All experiments are performed at room temperature unless otherwise indicated.

3.1 Random Conjugation

1. In a 1.5 mL or 2 mL microtube mix 1 mL of nanobody (1 mg/mL) with 4 molar equivalents of photosensitizer or fluorophore (pre-dissolved in DMSO) and incubate for 2 h at room temperature on an overhead shaker (*see* **Notes 1** and **2**). Protect the tube from light.
2. Separate nanobody-photosensitizer or nanobody-fluorophore conjugate from free photosensitizer or fluorophore by gel filtration chromatography using Zeba desalting columns in two sequential steps or dialysis (*see* **Notes 3** and **4**). To use 5 mL Zeba column (7 kDa cutoff), first remove the column's bottom closure and loosen the cap. Place the column in a 15 mL falcon tube and centrifuge at 1000 × *g* for 2 min at 4 °C to remove the storage buffer. Discard the buffer and add 2.5 mL of washing buffer (PBS 1x) on top of the resin. Using the centrifugation conditions indicated by the provider (i.e., 1000 × *g* for 2 min), rinse the resin three consecutive times, and then transfer the column into a clean 15 mL tube. Apply the sample on top of the column and collect the flow-through after centrifugation (1000 × *g*, 2 min, 4 °C).
3. Take 0.2–0.5 μL (corresponding approximately to 0.2–0.5 μg) of nanobody-photosensitizer or nanobody-fluorophore conjugate into a microtube and prepare the samples for gel electrophoresis with the sample buffer without bromophenol blue.
4. After boiling the samples for 5 min, load the samples on a 15% SDS-PAGE gel. Run the gel until a clear distance of 1–2 cm is left between the front of the gel and the end of the glass. Then, disassemble the gel cassette and place the gel immediately on the Odyssey infrared scanner (*see* **Note 5**).
5. Image the gel using the appropriate channel (700 or 800 nm) to detect the fluorescence and estimate the percentage of free photosensitizer fluorophore (fluorescence intensity of the band of free/total fluorescence intensity). The gel can be stained by Coomassie blue afterwards to visualize the protein bands (*see* **Note 6**) (Fig. 2).
6. If the percentage of free dye is higher than 10%, repeat the purification using Zeba columns and determine the purity of the conjugate on gel again (*see* **Note 7**).
7. Determine the DOC by using the formulas provided by the manufacturer of the photosensitizer or fluorophore. Determine the absorption of the sample at 280 nm and at the wavelength of the maximum absorption of the photosensitizer or fluorophore (i.e., 689 nm for IRDye 700DX, or 774 nm for IRDye 800CW). Use the function Uv-Vis of a NanoDrop spectrophotometer. Dilute the sample 1:3 and 1:6 in PBS and measure the absorbance of each sample twice. The

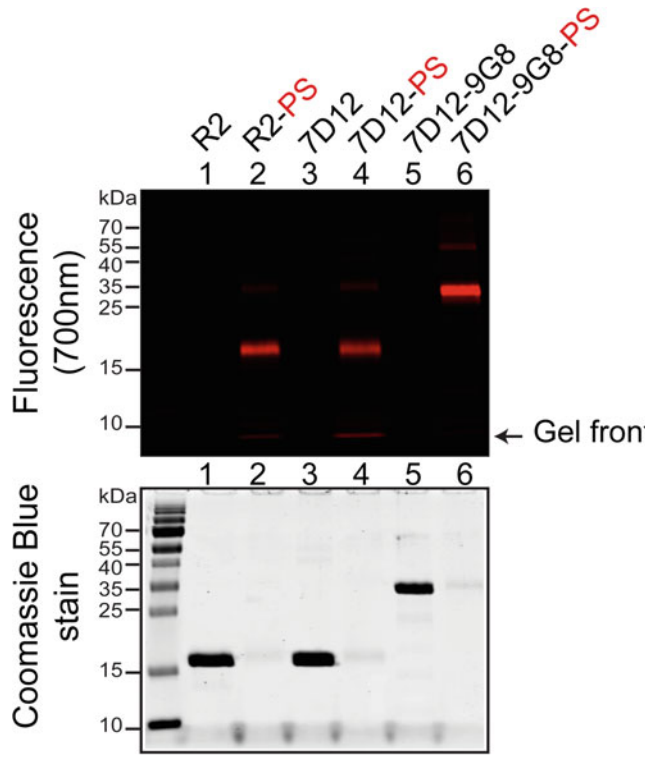

Fig. 2 Nanobodies and the respective nanobody-photosensitizer conjugates are separated by SDS-PAGE, with the following order (numbered 1–6): R2, R2–PS, 7D12, 7D12–PS, 7D12-9G8, and 7D12-9G8–PS. Prior to the Coomassie stain, the fluorescence of the photosensitizer is detected (depicted in red, top gel); after the Coomassie stain, the proteins are visualized (depicted in black, bottom gel). (Figure from [9])

concentration of protein and DOC can be calculated with the following formulas:

$$\text{Protein concentration}\left(\frac{\text{mg}}{\text{ml}}\right) = \frac{A280 - (R \times A)}{\text{Ext}} \times \text{Mw} \times \text{DF}$$

where R = correction factor (absorbance of photosensitizer/fluorophore at 280 nm), A = maximum absorbance of dye (λmax), Ext = extension coefficient of protein ($M^{-1}\,cm^{-1}$), Mw = molecular weight of protein, DF (dilution factor) = the factor by which the conjugate was diluted before measuring with Nanodrop

$$\frac{\text{Dye}}{\text{Protein}}\text{ratio} = \frac{A}{\text{Extdye}} \div \frac{A280 - (R \times A)}{\text{Extprot}}$$

where Extdye = extension coefficient of photosensitizer/fluorophore ($M^{-1}\,cm^{-1}$), R = correction factor (absorbance of dye at 280 nm), Extprot = extension coefficient of protein ($M^{-1}\,cm^{-1}$)

8. Depending on the subsequent experiments planned, it may be necessary to have a solution of nanobody-photosensitizer or nanobody-fluorophore conjugate with higher concentration. In that case, use Amicon centrifugal filters to concentrate the sample. Depending on the sample volume, 0.5 ml or 4 ml tubes (10 kDa cutoff) can be used.

   The following is a general procedure for using 0.5 mL tube:

   In order to remove the trace of glycerine and also pre-wet the tube, add PBS to the Amicon tube and centrifuge at 14,000 × *g*, 10 min, 4 °C. Add the conjugate solution to the tube and centrifuge at 14,000 × *g*, 10 min, 4 °C. Place the filter upside down in a clean tube and centrifuge at 1000 × *g*, 2 min, 4 °C, to collect the concentrated sample.

   The following is a general procedure for using 4 mL tube (10 kDa):

   In order to remove the trace of glycerine and also pre-wet the tube, add PBS to the Amicon tube and centrifuge at 5000 × *g*, 10 min, 4 °C. Add the conjugate solution to the tube and centrifuge at 5000 × *g*, 4 °C, for a maximum 15–60 min. Use a Pasteur pipette to collect the concentrated sample from the bottom of the filter.

3.2 Site-Directed Conjugation

Two different protocols for site-directed conjugation are presented here. The optimal method leading to the highest DOC should be determined experimentally for each nanobody.

3.2.1 Protocol 1

1. Replace the buffer in which the nanobody is dissolved (i.e., PBS) with Tris buffer, using Zeba desalting column (*see* Subheading 3.1, **step 2**, for the procedure).
2. Add 0.5 M of TCEP to the nanobody solution to a final concentration of 20 mM and incubate for 15 min at room temperature, to reduce possible disulfide bonds between the C-terminal cysteines. No shaking is needed (*see* **Notes 8–10**).
3. To stop this reduction step, exchange the buffer to PBS-EDTA using Zeba column (*see* Subheading 3.1, **step 2**, for the procedure; *see* **Note 11**).
4. Use the NanoDrop spectrophotometer to determine the absorption at 280 nm and thus the protein concentration (by correcting with the known extinction coefficient of the protein), and adjust the concentration of the protein to 1 mg/mL (*see* **Note 2**).
5. Immediately mix the nanobody with 3 molar equivalents of photosensitizer or fluorophore and incubate overnight at 4 °C on an overhead shaker (*see* **Note 11**). Protect the reaction tube from light.

6. If the overnight conjugation mixture looks cloudy, first spin down at 1000 × *g* for 1 min and proceed with the supernatant. Remove the free dye using two consecutive Zeba desalting columns (*see* Subheading 3.1, **step 2**, for the procedure). Proceed with the protocol as in Subheading 3.1 from **step 3**.

3.2.2 Protocol 2

1. Add 0.5 M of TCEP to the nanobody solution to a final concentration of 20 mM and incubate for 15 min at room temperature, to reduce possible disulfide bonds between the C-terminal cysteines. No shaking is needed (*see* **Notes 8–10**).
2. To stop this reduction step, exchange the buffer to sodium phosphate containing NaCl and EDTA, using Zeba desalting column (*see* Subheading 3.1, **step 2**, for the procedure, **Note 11**). Proceed with the protocol as in Subheading 3.2 from **step 4**.

4 Notes

1. The nanobody-photosensitizer or fluorophore molar ratio needs to be optimized for each specific nanobody.
2. The recommended starting concentration of nanobody for random and site-directed conjugation is 1 mg/mL. However, higher concentration can be used in order to obtain higher DOC. Extra caution should be taken into account as this could compromise the binding properties of the conjugate to the target of interest.
3. The number of Zeba columns used for purification of the conjugate can vary, with the photosensitizer or fluorophore employed, as well as the nanobody sequence; thus, this should be explored experimentally.
4. Performing dialysis for removing the free photosensitizer or fluorophore only works for the most hydrophilic molecules.
5. A pre-stained ruler will indicate the position of the front of the gel. When disassembling the cassette to image the gel, do not place the gel in any buffer. Instead, place the gel from the cassette immediately on the scanner. Placing the gel in buffer could lead to the diffusion of unconjugated photosensitizer or fluorophore from the front of the gel, which would lead to an inaccurate determination of the percentage of free photosensitizer or fluorophore in the sample.
6. In general, 1–2 μg of protein can be stained with Coomassie blue for a well-visible protein band. Lower amounts of protein are usually loaded for conjugates, which may not be well visible with Coomassie staining, but it is possible to load side-by-side samples of purified protein and conjugate.

7. If the reaction is performed in large scale (>1 mL), initially 5 mL Zeba columns can be used for purification. Based on our experience, if the amount of free dye is still more than 10% after 2 × 5 mL Zeba columns, a good purification is obtained with subsequent concentration of the sample using Amicon centrifugal filters, followed by 2 mL Zeba columns.
8. TCEP can be purchased as aqueous solution in ampule (0.5 M) or powder. In general, TCEP is very soluble in aqueous buffers at almost any pH. However, it is significantly less soluble in organic solvents such as methanol and ethanol.
9. For most applications, 5–50 mM TCEP effectively reduces disulfide bonds in peptides or proteins within a few minutes at room temperature.
10. There are studies suggesting that the reaction can be performed in the presence of TCEP since it does not contain thiol groups. However, some groups have reported that TCEP can react with the maleimide group during labeling reaction [12]. Therefore, it is recommended to remove TCEP before the reaction with the photosensitizer or fluorophore.
11. Site-directed conjugation in PBS works well, but other buffers with pH from 6.5 to 7.5 can also be used. Since unprotonated amines can also react with maleimides, reaction above pH 8.0 should be avoided. The reaction can be carried out at room temperature for 2 h or at 4 °C for 16–18 h. For heat-resistant nanobodies, the reaction can be performed at 37 °C for 2 h.

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).

References

1. Hofman EG, Ruonala MO, Bader AN et al (2008) EGF induces coalescence of different lipid rafts. J Cell Sci 121:2519–2528
2. Heukers R, Vermeulen JF, Fereidouni F et al (2013) Endocytosis of EGFR requires its kinase activity and N-terminal transmembrane dimerization motif. J Cell Sci 126:4900–4912
3. Oliveira S, van Dongen GAMS, Walsum MS et al (2012) Rapid visualization of human tumor xenografts through optical imaging with a near-infrared fluorescent anti–epidermal growth factor receptor nanobody. Mol Imaging 11:33–46
4. van Driel PBAA, van der Vorst JR, Verbeek FPR et al (2014) Intraoperative fluorescence delineation of head and neck cancer with a fluorescent anti-epidermal growth factor receptor nanobody. Int J Cancer 134:2663–2673
5. Kijanka M, Warnders F-J, el Khattabi M et al (2013) Rapid optical imaging of human breast tumour xenografts using anti-HER2 VHHs site-directly conjugated to IRDye 800CW for

image-guided surgery. Eur J Nucl Med Mol I 40:1718–1729

6. Kijanka MM, van Brussel ASA, van der Wall E et al (2016) Optical imaging of pre-invasive breast cancer with a combination of VHHs targeting CAIX and HER2 increases contrast and facilitates tumour characterization. EJNMMI Res 6:1–13
7. van Brussel ASA, Adams A, Oliveira S et al (2016) Hypoxia-targeting fluorescent Nanobodies for optical molecular imaging of pre-invasive breast cancer. Mol Imaging Biol 18:535–544
8. Heukers R, van Bergen en Henegouwen PMP, Oliveira S (2014) Nanobody–photosensitizer conjugates for targeted photodynamic therapy. Nanomed Nanotechnol 10:1441–1451
9. van Driel PBAA, Boonstra MC, Slooter MD et al (2016) EGFR targeted nanobody–photosensitizer conjugates for photodynamic therapy in a pre-clinical model of head and neck cancer. J Control Release 229:93–105
10. Heukers R, Mashayekhi V, Ramirez-Escudero M et al (2019) VHH-photosensitizer conjugates for targeted photodynamic therapy of Met-overexpressing tumor cells. Antibodies 8: 1–13
11. de Groof TWM, Mashayekhi V, Fan TS et al (2019) Nanobody-targeted photodynamic therapy selectively kills viral GPCR-expressing glioblastoma cells. Mol Pharm 16:3145–3156
12. Kim Y, Ho SO, Gassman NR et al (2008) Efficient site-specific labeling of proteins via cysteines. Bioconjug Chem 19:786–791