Supporting Information

Targeted Luminescent NIR Polymer-Nanoprobes for In Vivo Imaging of Tumor Hypoxia

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SI Materials and Methods

Chemicals. 2-Iminothiolane (Traut's reagent), 5,5'-dithio-bis(2-nitro-benzoic acid) (Ellman's reagent), cysteine, sodium phosphate, ethylenediaminetetraacetic acid (EDTA), sodium sulfite, and poly-L-lysine were purchased from Sigma-Aldrich (Germany). NaCl, EDTA, Tris/HCl, Triton X-100 were obtained by Carl Roth (Germany). All reagents were of analytical grade and used as received. All solvents (tetrahydrofuran (THF), ethanol and dimethylformamide (DMF) purchased from Sigma-Aldrich, were of UV-spectroscopic grade and used as received. DMEM and RPMI 1640 medium, 2-mercaptoethanol, L-glutamine, fetal calf serum (FCS), phosphate buffered saline (PBS) and Prolong Gold Antifade Reagent with DAPI were obtained from Invitrogen (Germany).

Preparation of Her2/neu-targeting Ox-PS-NPs. For the labeling of the amino-modified polystyrene particles we altered a prior described method,[47] initially used for the labeling of nanoparticles consisting of human serum albumin.

Herceptin was dialyzed using Slide-A-Lyzer Dialysis Cassettes (20000 MWCO). For the preparation of 20 mg Herceptin-labeled Ox-PS-NPs, the cassette was filled with 1 mg herceptin dissolved in 0.3 mL dialysis buffer (0.1 M NaH_2PO_4, 0.01 M EDTA, pH 8, N_2-purged) and dialyzed against 0.5 L well-stirred dialysis buffer at 4 °C. After dialysis, the content of the dialysis cassettes was diluted to 1 mL with N_2-purged dialysis buffer. Under N_2 and constant stirring, herceptin (1 mg, ca. 7 µM) was thiolated by addition of 50 µL freshly prepared 7 mM 2-iminothiolane in phosphate buffer. The reaction mixture was shaken for 2 h at 600 rpm. Subsequently, the thiolated anibody was purified by size exclusion chromatography on a PD-10 column eluting with dialysis buffer.

The herceptin concentration used for particle modification after antibody thiolation was determined photometrically (ε_{280nm} = 150000 L×mol^{-1}×cm^{-1}) yielding a concentration of 6 µM of thiolated herceptin. The degree of thiolation was obtained with Ellman's reagent ($\varepsilon_{412\ nm}$ = 14150 L×mol^{-1}×cm^{-1}). For this purpose, 2 µL of Ellman's reagent (4 mg/mL in phosphate buffer) were added to 250 µL column eluent, incubated for 15 min at room temperature, and then, the absorbance at 412 nm was determined and used for the calculation of the number of thiol groups. We found 7 µM thiol groups equaling an average of about 1 thiol group per herceptin molecule.

For coupling of the thiolated herceptin to the PEG-lated Ox-PS-NPs, 20 mg Ox-PS-NPs-PEG-Mal in 1 mL phosphate buffer were mixed with 1 mL of thiolated herceptin (6 µM). The reaction mixture was shaken for 16 h at room temperature. 10 mg cysteine in 100 µL phosphate buffer were added and incubated for 30 min to block residual maleimide functionalities. Subsequently, the herceptin-PS-NPs were purified twice by repetitive rounds of centrifugation, removal of supernatant, and PSP resuspension in 1 mL sterile water.

The amount of PS-NPs-bound herceptin was determined using a BCA Protein Assay following the manufacturer's instructions. Typically, 50 µL herceptin-PS-NPs containing suspension was used and the absorption spectra of the particle-containing assay were measured. The absorbance background, consisting of absorbance of the incorporated dyes and scattering of the PS-NPs was removed by subtracting the spectra of equally stained and concentrated PS-NPs in water. We found an average of 10 herceptin molecules per 100 nm particle.

Integrating sphere setup. This custom-made setup consists of a xenon lamp coupled to a single monochromator, a 6 inch Spectraflect-coated integrating sphere (Labsphere GmbH) coupled with a quartz fiber to an imaging spectrograph (Shamrock 303i, Andor Inc.) equipped with a Peltier-cooled thinned back side illuminated deep depletion charge coupled device. A

reference detector was implemented into the setup to account for fluctuations of the spectral radiant power reaching the sample. Prior to the Φ_f measurements, the wavelength accuracy, the linearity, and the spectral responsivity $s(\lambda_{em})$ of the integrating sphere-detection system ensemble were determined following previously described procedures.[42,48-49] For the Φ_f measurements, the sample (quartz cell filled with PSP suspension) or blank (solvent-filled quartz cell) were mounted in the center of the integrating sphere and directly illuminated. The excitation light (here 620 nm) was focused into the middle of the sample. The absolute photoluminescence QY was then calculated from the measured spectrally corrected signals of the blank (I_{CB}) and the sample (I_{CS}) according to equation 1. [50] The measured fluorescence quantum yields were corrected for reabsorption effects. [42,48-49]

$$\Phi_f = \frac{\int_{\lambda_{em_1}}^{\lambda_{em_2}} \frac{\lambda_{em}}{hc}\left(I_{CS}(\lambda_{em}) - I_{CB}(\lambda_{em})\right) d\lambda_{em}}{\int_{\lambda_{ex_1}}^{\lambda_{ex_2}} \frac{\lambda_{ex}}{hc}\left(I_{CB}(\lambda_{ex}) - I_{CS}(\lambda_{ex})\right) d\lambda_{ex}} = N_{em} / N_{abs} \qquad \text{(eq. 1)}$$

Calibration of oxygen response. Effective oxygen concentrations of sodium sulfite solutions were measured parallel to spectroscopy. For this purpose, 100 µL of each solution and 3 µL of PBS (140 mM) were pipetted into the OxoPlate® and sealed with 100 µL of paraffin oil. The plate was covered with a standard 96 well plate cap (Greiner) and read out with a plate reader (Labsystems Fluoroskan Ascent). The emission spectra and the oxygen concentration in the wells were measured simultaneously at 37 °C.

Fluorescence lifetime imaging. Optical set-up: The excitation light was focused onto the sample using a PCX 18_18 MgF2 TS lens (Edmund Optics, Karlsruhe, Germany) filtered through a BG 12 filter (Schott; Mainz, Germany) with a thickness of 2 mm, and the emission was detected through a RG 695 long pass filter (AHF Analysentechnik; Tübingen, Germany).

Procedure: A 3 × 3 cm sample of Mylar® foil coated with the 6 µm PS film was placed in a calibration chamber. The sample was excited by a light pulse from a 405 nm-LED and the luminescence intensity was measured in two precisely timed gates by a triggered CCD camera followed by measurement of a dark image for background subtraction. The excitation time was 40 µs, the time gate 1 was opened after 1 µs, and time gate 2 after 21 µs, with gate widths of 20 µs.

Fluorescence lifetime calibration of oxygen response. The *p*/*T* calibration chamber was provided by the German Aerospace Center (DLR, Göttingen, Germany). All time-resolved measurements were performed with an Imagex TGI 14 bit b/w CCD camera from Photonic Research Systems equipped with a Schneider-Kreuznach Xenon 0.95/17 lens (Jos. Schneider Optische Werke, Bad Kreuznach, Germany) and a 405 nm-LED from Roithner Lasertechnik (Vienna, Austria).

The dependence between the luminescence lifetime (τ) of PdTPTBP and oxygen partial pressure was investigated in a knife-coated PS film of approximately 6 µm thickness. Referenced images were obtained via the rapid lifetime determination (RLD) method.[44] The imaging and data acquisition process has been previously described.[45]

Western blot. Cells were plated on Petri dishes, one dish for each setting, and exposed to the experimentally relevant oxygen concentrations. After 4.5 h, the cells were washed with PBS and 500 µl of lysis buffer (400 mM NaCl, 1mM EDTA, 10 mM Tris/HCl pH 8.0 and 0.1% Triton X-100) supplemented with Complete Mini Protease Inhibitor Cocktail Tablets. Lysed cells were scraped and cell debris removed by centrifugation. The protein concentration was measured using Bradford reagent. 100 µg of total cellular proteins were separated by 10% SDS-PAGE and detected by immunoblotting. Rabbit anti-mouse HIF-1α and mouse anti-ß-

actin antibodies were used. The immunocomplexes were detected using horseradish-conjugated goat anti-rabbit or goat anti-mouse antibodies and incubation of the membranes with 100 mM Tris-HCl, pH 8.5, 2.65 mM H_2O_2, 0.45 mM luminol, and 0.625 mM coumaric acid for one minute. The luminescence was visualized using the LAS 300 camera system (Fuji).

NIR microscopy filters. The Zeiss microscope was equipped wit thte following filters: BP 620/60 excitation filter, FT 662 dichroic mirror, and BP 809/91 emission filter for PdTPTBP and BP 640/30 excitation filter, FT 660 dichroic mirror and BP 690/50 for DY-635. DAPI fluorescence was acquired using BP 635/25 excitation filter, FT 395 dichroic mirror, and a BP 445/50 emission filter.

A threshold value was applied for each single image as a mean background value in a selected cell-free region including a twofold standard deviation to remove background fluorescence.

In vitro oxygen imaging. Intracellular oxygen-sensing was evaluated with cells plated onto poly-L-lysine-coated coverslips at a density of 50000 cells pro 3.3 cm². After 2 days, the cells were placed in an oxygen-controlled work station (InVivo 400; Ruskin, Bridgend, UK) in either 5% or 1% O_2 adjusted with N_2. Ox-PS-NPs (0.5 µg/ml) were added directly to 200 µl culture medium. After 4.5 h cells were washed 3-4 times with PBS, fixed for 5 min in 4% PFA/PBS, and mounted with Prolong Gold Antifade Reagent with DAPI. The mounted glass slides were exposed over night to the experimental oxygen concentrations (either 1% or 5%) and afterwards additionally sealed with nail polish.

To study binding and internalization behavior of herceptin-functionalised PS-NPs, SK-BR-3 and MDA-MB-231 cells were plated on poly-L-lysine-coated coverslips at a density of 50000 cells pro 3.3 cm² and allowed to attach for at least 24h. PS-NPs were added directly to the cell-

culture medium (0.5 µg/µl in 200µl volume) and incubated for 1.5 - 24 h. Afterwards, cells were washed and fixed as described above.

NIR fluorescence imaging. Response of the Ox-PS-NPs to the decreasing oxygen concentration was measured as a ratio between average fluorescence intensity of PdTPTBP and DY-635 for each well. All measurements were performed in triplicates. Data analysis was performed with the OPTX-OptiView-2-02-00 analysis software (ART).

In vivo imaging. The Optix MX2 scanner is suitable for intensity imaging as well as fluorescence lifetime imaging studies in the nanosecond time-domain. It is equipped with four laser diodes (635 nm, 670 nm, 730 nm, and 780 nm). DY-635 and PdTPTBP fluorescence was measured upon excitation at 635 nm using a 670 nm +/-20 emission filter for DY-635 and a 700 nm long-pass emission filter for PdTPTBP. Scans were obtained with 1.0 mm raster and photon collection time (so called integration time) of 0.7 s per scan point. The fluorescence intensity was displayed in normalized counts (NC) where the measured fluorescence intensity (counts) is normalized for varying laser power and integration times, allowing comparison of measurements with different instrument settings.

Figures and Tables:

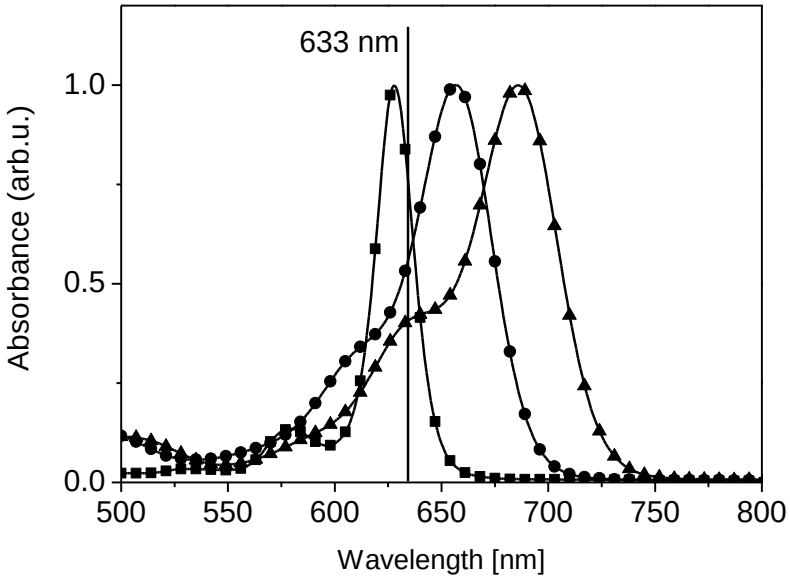

Figure 1S. Normalized absorption spectra of PdTPTBP (squares) and DY-635 (circles) in DMF.

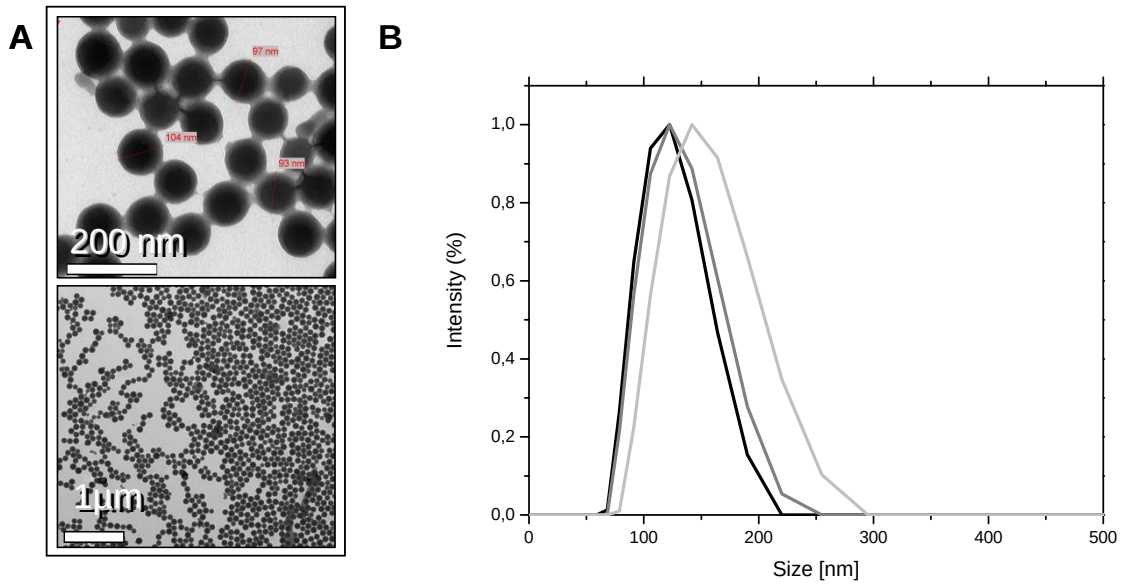

Figure 2S. A: Representative TEM images of PdTPTBP- and DY-635-doped 100 nm PS-NPs (Ox-PS-NPs) B: DLS data of 100 nm PS-NPs: amino-modified and blank (black), amino-modified and doped with PdTPTBP and DY-635 (dark grey) and herceptin-modified and doped with PdTPTBP and DY-635 (light grey).

Figure 3S

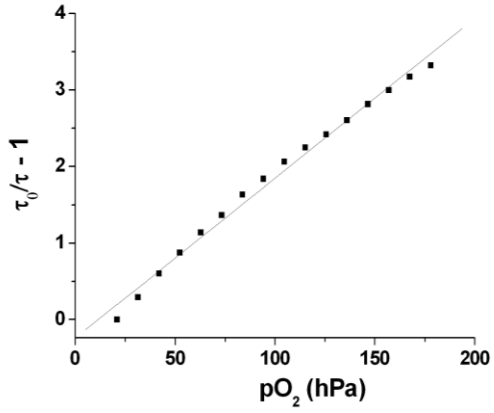

Fig. 3S. Change of phosphorescence lifetime of PdTPTBP in PS vs. pO_2 displayed as Stern-Volmer plot (τ_0 / τ) with linear fit (R^2 = 0.990).

Figure 4S

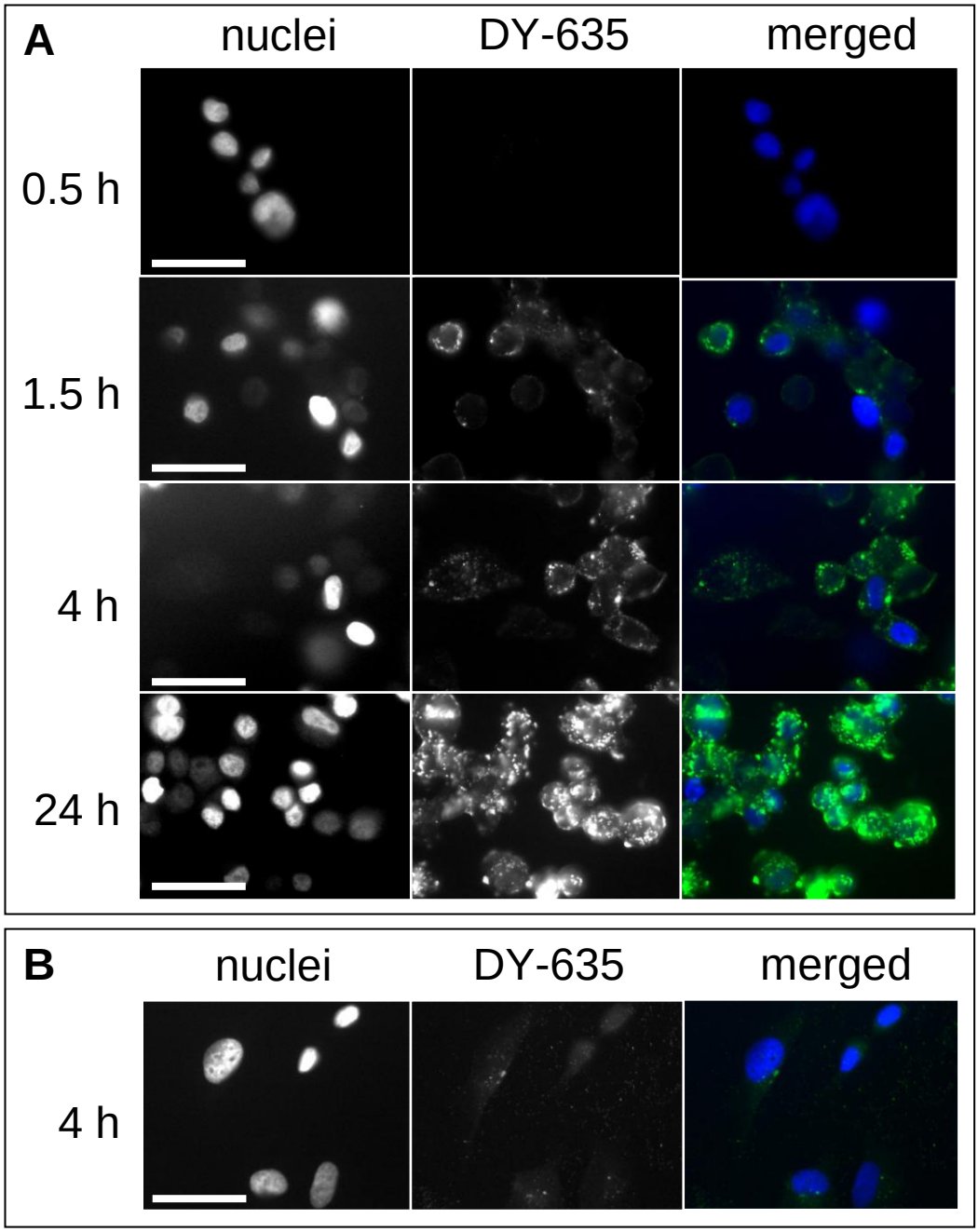

Figure 4S. Binding of herceptin-functionalized PS-NPs to (A) the Her2/*neu*-expressing mammary carcinoma SK-BR-3 cells, and (B) Her2/*neu*-negative mammary carcinoma MDA-MB-231 control cells. Blue: nuclei, green: DY-635. Bar: 50 µm.

Table 1S. Ratiometric response of Ox-PS-NPs to decreasing oxygen concentrations *in vitro*.

O_2 concentration	Mean Ratio ± SD*	Ratio range
20%	1.10 ± 0.04	0.64 - 1.79
1%	1.71 ± 0.18	0.76 - 3.93
1% - 20%	1.01 ± 0.04	0.49 - 1.70
5%	1.44 ± 0.11	0.78 - 3.14
5% - 20%	1.06 ± 0.32	0.63 - 2.47

*SD = standard deviation