

Controlling aminosilane layer thickness to extend plasma half-life of stealth persistent luminescence nanoparticles *in vivo*

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EXPERIMENTAL PROCEDURES.

Chemicals. (3-Aminopropyl)-triethoxysilane (99%) was obtained from Sigma-Aldrich. Zinc nitrate hexahydrate (>99%) was purchased from Fluka. Gallium oxide (99.999%) and chromium (III) nitrate nonahydrate (99.99%) were purchased from Alfa Aesar. Dimethylformamide (>99.9%) was purchased from SDS. Alpha-methoxy-omega-N-hydroxysuccinimide poly(ethylene glycol) PEG MW 5.000 Dalton was bought from Iris Biotech GmbH.

Preparation of ZGO nanoparticles. $\text{ZnGa}_{1.995}\text{Cr}_{0.005}\text{O}_4$ nanoparticles were synthesized by hydrothermal method and low-temperature sintering in air. First, gallium nitrate was formed by reacting 8.94 mmol of gallium oxide with 10 mL concentrated nitric acid (35 wt%) under hydrothermal condition at 150°C overnight. Then, a mixture of 0.04 mmol of chromium nitrate and 8.97 mmol of zinc nitrate in 10 mL of water was added to the previous solution of gallium nitrate under vigorous stirring. The resulting solution was adjusted to pH 7.5 with an ammonia solution (30 wt%), stirred for 3 hours at room temperature, and transferred into a 25 mL Teflon-lined stainless steel autoclave for 24h heat treatment at 120°C. The resulting compound was washed several times with water and ethanol before drying at 60°C for 2 hours. The dry white powder was finally sintered in air at 750°C for 5 hours. Hydroxylation was performed by basic wet grinding of the powder (500 mg) for 15 minutes, with a mortar and pestle in 50 mL of 5 mM NaOH solution, and overnight vigorous stirring of the resulting

suspension at room temperature. Nanoparticles with a diameter of 85 nm were first selected from the whole polydisperse colloidal suspension by centrifugation on a SANYO MSE Mistral 1000 at 4500 rpm for 5 minutes. They were located in the supernatant (assessed by Dynamic Light Scattering). The supernatants were gathered and concentrated to a final 5 mg/mL suspension.

Nanoparticles functionalization. ZGO-OH nanoparticles were coated according to slightly modified existing protocols. Briefly, ZGO-NH₂ nanoparticles were obtained by reacting 3-aminopropyl-triethoxysilane (APTES) with ZGO-OH at the concentration of 2 mg/mL. The solvent, APTES concentration, and reaction time were adjusted to provide the desired surface states. ZGO-NH₂ (DMF/3h), respectively ZGO-NH₂ (DMF/6h), were obtained by reacting ZGO-OH nanoparticles, dispersed in DMF, and APTES at the concentration of 1% v/v for 3 hours, respectively 6 hours, at room temperature. Particles were washed from the unreacted APTES by three centrifugation and redispersion steps in DMF. ZGO-PEG nanoparticles were obtained by reacting 10 μmol of MeO-PEG_{5kDa}-NHS (50 mg) with 5 mg of each ZGO-NH₂ nanoparticles dispersed in DMF at the concentration of 2 mg/mL. To ensure a maximum PEG density, the last functionalization step was achieved overnight, under vigorous stirring at 90°C.

Nanoparticles characterization. X-rays diffraction patterns were obtained on a Panalytical X'Pert Pro diffractometer with an incident-beam Ge monochromator, at $U = 45$ kV and $I = 40$ mA. The photoluminescence excitation spectrum was recorded on a Varian Cary Eclipse Fluorescence spectrophotometer at room temperature. Persistent luminescence emission spectrum was measured after 2 minutes excitation under the orange/red LEDs source (Bridgelux). Light was collected via an Acton SpectraPro monochromator coupled with a Princeton CCD camera cooled at -70°C.

ZGO nanoparticles were characterized using transmission electron microscopy (JEOL JEM-100S) and dynamic light scattering and zeta potential measurements in 20 mM NaCl, performed on a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA) equipped with a 632.8 nm helium neon laser and 5-mW power, with a detection angle at 173° (non-invasive backscattering).

Thermogravimetric analysis (TGA) was performed using a Setaram Setsys evolution 1600 (Argon atmosphere, temperature range: from 20°C to 780°C, 10°C/min) on 10 mg dry samples of 120 nm core PLNP, at each functionalization step.

The FTIR spectra of ZGO nanoparticles with different surface states were recorded by a Fourier transform infrared spectrophotometer (IRAffinity-1, Shimadzu).

Adsorption of Mouse Serum Proteins and Purification. Nanoparticles at the concentration of 1mg/mL were incubated for two hours at 37°C with 50% v/v mouse serum diluted in normal saline solution. Nanoparticles were washed from unbound proteins by several centrifugation steps (13 200 rpm for 10 minutes at room temperature).

Bradford Assay. Aliquots of 10 µL of each type of ZGO nanoparticles suspended in 5% glucose (20 µg), previously incubated with 50% v/v murine serum, were transferred to a 96-well plate, along with 10 µL aliquots of a bovine serum albumin (BSA) serial dilution. Next, 200 µL of Coomassie blue dye reagent (Bio-Rad) was added to each well, and the plate was incubated at 37°C for 10 minutes. Absorbance at 595 nm was measured using a plate reader (Wallac Victor² Multilabel Counter, Perkin Elmer). The BSA standard was prepared with ZGO-OH nanoparticles in order to take into account the absorbance of free nanoparticles.

Polyacrylamide Gel Electrophoresis (PAGE). Aliquots of 10 µL of each type of ZGO nanoparticles, previously incubated with 50% v/v murine serum, were mixed with 5 µL of 4x LDS sample buffer (Invitrogen), 2 µL of 10 x reducing agent (Invitrogen) and incubated at 90°C for 10 minutes. Samples, along with 5 µL of SeeBlue Plus2 Pre-Stained Standard (Invitrogen), were loaded on a 10% Bis-Tris gel in MOPS running buffer (Invitrogen) and resolved at 200 V for 60 minutes. After electrophoresis, the gel was fixed with 10% v/v acetic acid in 40% v/v methanol for 30 minutes and stained with silver (Bio-Rad) according to the manufacturer's protocol.

In vivo systemic injections. Five weeks old female BALB/c mice (Janvier, Le Genest St. Isle, France) were anesthetized by i.p. injection of a mixture of ketamine (85.8 mg/kg, Centravet, Plancoët, France) and xylazine (3.1 mg/kg, Bayer, Leverkusen, Germany) diluted in 150 mM NaCl. Systemic injections of

10^{13} ZGO nanoparticles, dispersed in 5% sterile glucose solution were then realized to perform imaging studies.

Imaging. Signal acquisition was carried out using a photon-counting system based on a cooled GaAs intensified charge-coupled device (ICCD) camera (Photon-Imager, Biospace, Paris, France). The ICCD aperture time was set to three minutes. ZGO nanoparticles (10^{13} in sterile 5% Glucose solution) were first excited *ex vivo* for 2 minutes under UV light (6W mercury discharge 254 nm lamp) before injection to mice via the caudal vein. Animals were then placed on their back under the photon-counting device, and the signal acquisitions were performed. After a 3 hours period and complete persistent luminescence extinction, the orange/red LEDs source was shined on the animal for two minutes to re-activate the persistent luminescence from ZGO nanoparticles, and the signal acquisition was resumed. Semi-quantization was achieved through the use of Biospace developed software, PhotoVision+. Experiments were conducted in agreement with a regional ethic committee for animal experimentation.

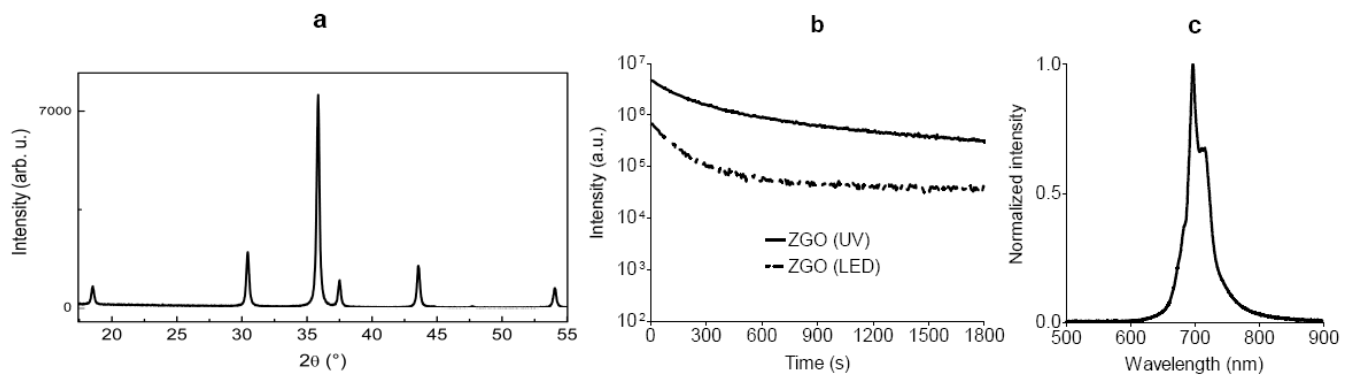


Figure S1. Physical characteristics of the ZGO compound. **a**, X-rays diffraction pattern. **b**, Persistent luminescence decay curves. **c**, Persistent luminescence emission spectrum.

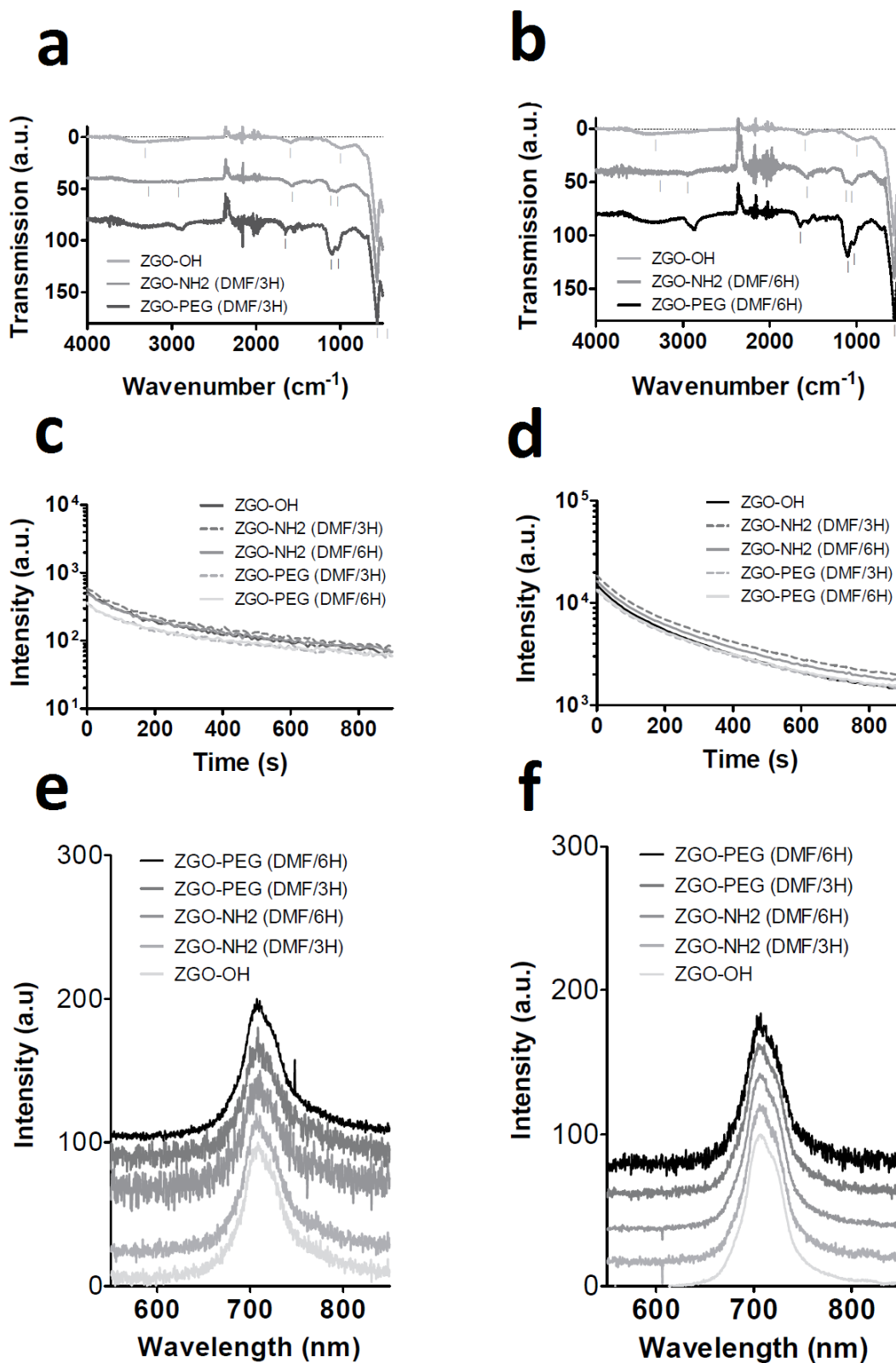


Figure S2. Physical characteristics of ZGO nanoparticles with different surface states. **a-b**, FTIR spectra of different ZGO nanoparticles, major bands are represented as bold dashes. **c**, Persistent luminescence decay curves of different ZGO nanoparticles following visible activation. **d**, Persistent luminescence decay curves of different ZGO nanoparticles following UV activation. **e**, Persistent

luminescence emission spectrum of different ZGO nanoparticles following visible activation. **f**, Persistent luminescence emission spectrum of different ZGO nanoparticles following UV activation.

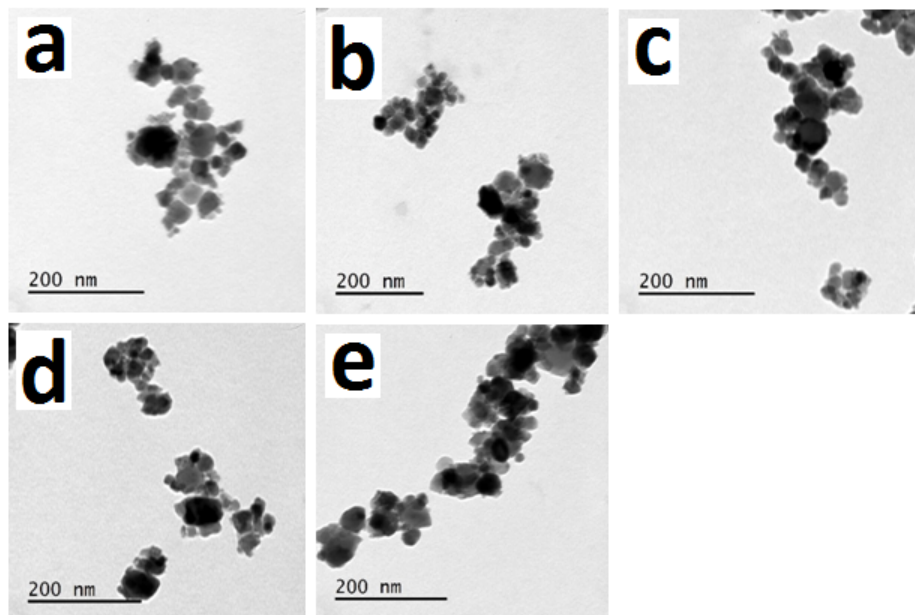


Figure S3. Transmission electron micrographs of ZGO nanoparticles with different surface states. **a**, ZGO-OH. **b**, ZGO-NH₂ (DMF/3h). **c**, ZGO-NH₂ (DMF/6h). **d**, ZGO-PEG (DMF/3h). **e**, ZGO-PEG (DMF/6h).

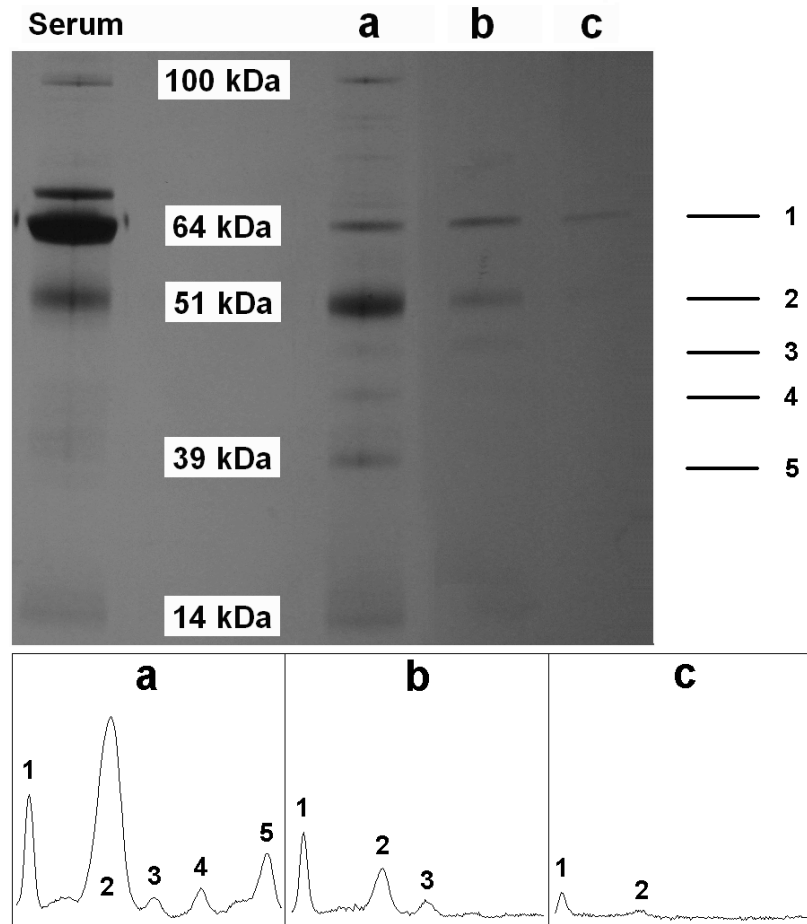


Figure S4. Polyacrylamide gel electrophoresis analysis with ImageJ software.

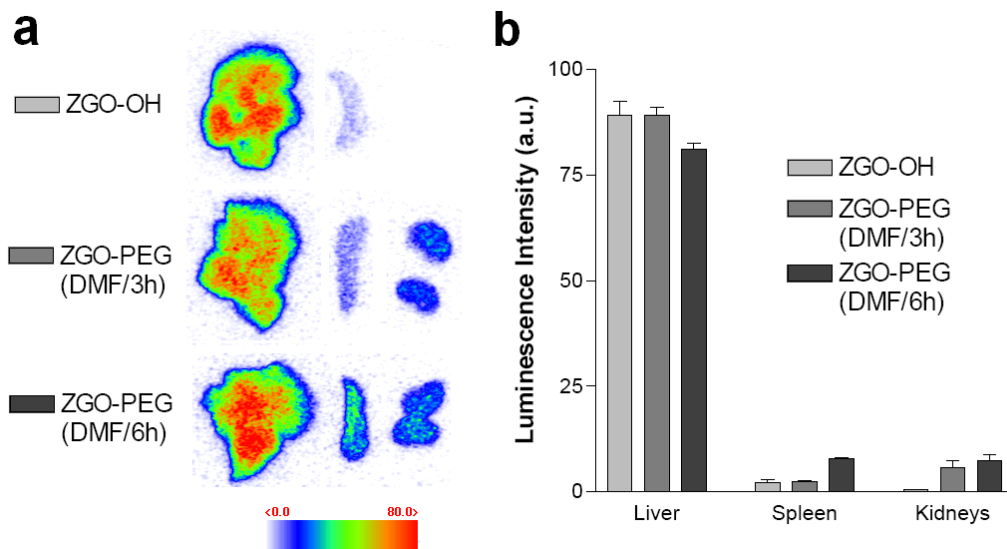


Figure S5. 24 hours *ex vivo* biodistribution of ZGO-PEG nanoparticles (n = 3). **a**, Persistent luminescence images recorded after 2 minutes excitation of the harvested organs under the red-LEDs source. **b**, Image-based quantitative biodistribution of ZGO-PEG nanoparticles.