Supplementary materials

Ultrasmall AGuIX Theranostic Nanoparticles for Vascular-Targeted Interstitial Photodynamic Therapy of Gliobastoma

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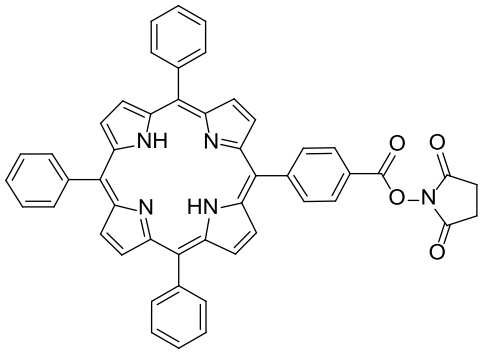
1. Supplemental methods

1.1. Peptides characterizations

1.1.1. General

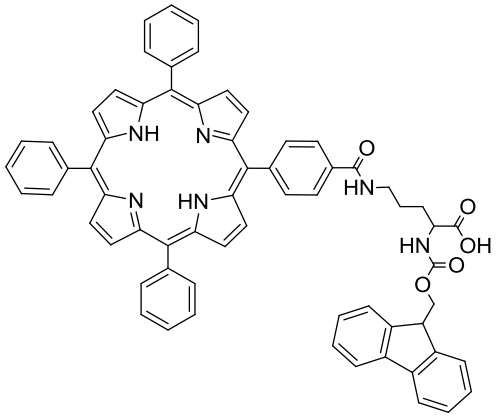
All reactions involving porphyrin compounds were performed in the dark. Reactions were monitored by thin-layer chromatography (TLC) using aluminium-backed silica gel plates (Macharey-Nagel ALUGRAM® SIL >G/UV254). TLC spots were viewed under ultraviolet light. NMR spectra (^{1}H, COSY and TOCSY) were recorded on a BRUKER AVANCE spectrometer at 300 MHz. The spectra were recorded in $CDCl_3$ and DMSO-d_6 solvents at room temperature (T = 298 K) using TMS (δ = 0 ppm) or DMSO residual peak (δ = 2.5 ppm) as internal references respectively. Chemical shifts (δ) are given in parts per million (ppm), and coupling constants (J) are given in hertz (Hz). Multiplicities are reported as follow: s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet, br = broad and Ar. = aromatic. Electron spray ionization mass spectra (ESI-MS) were recorded on a Brucker MicroTof-Q HR spectrometer in the "Service commun de Spectrométrie de Masse", Faculté des Sciences et Technologies (Vandoeuvre-lès-Nancy, France). Absorption spectra were recorded on a Perkin-Elmer Lambda EZ210 (Courtaboeuf, France) double beam UV-visible spectrophotometer. Fluorescence spectra were recorded on a Fluorolog-3 spectrofluorimeter FL3-222 (Horiba Jobin Yvon, Longjumeau, France) with a thermostated cell compartment (25 °C) using a 450 W Xenon lamp. Fluorescence quantum yield (ϕ_F) were determined using tetraphenyl porphyrin (TPP) solution in toluene as fluorescence standard (ϕ_F = 0.11) (1). Singlet oxygen quantum yield (ϕ_Δ) were determined by direct measurement of the infrared luminescence using Rose Bengal in EtOH as a reference (ϕ_Δ = 0.68) (2). The absorbance value at the excitation wavelength of the reference and the sample solutions were set around 0.2.

1.1.2. PS-NHS: 5-(4-carboxyphenylsuccinimide ester)–10,15,20–triphenylporphyrin



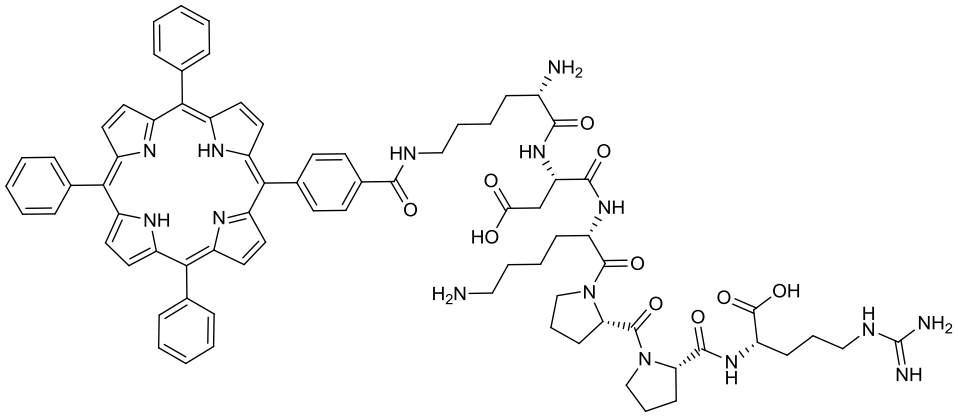
R_f = 0.79 (CH_2Cl_2/EtOH = 97:3, v/v). ^{1}H NMR (300 MHz, DMSO-d_6, δ): -2.91 (s, 2H, NH), 3.00 (s, 4H, CH_2), 7.84 (s, 9H, $H_{m\text{- and }p\text{-phenyl}}$), 8.22 (d, 6H, $H_{o\text{-phenyl}}$), 8.50 (dd, 4H, $H_{o\text{-phenyl -COOH}}$), 8.85 (s, 8H, $H_{\beta\text{-pyrrole}}$). HRMS (ESI+): *m/z* calcd. for $C_{49}H_{33}N_5O_4$ $[M - H]^+$ 756.2605; found 756.2588. UV/Vis (EtOH*): λ_{max}*(log ε) = 419 (5.70), 512 (4.35), 545 (3.99), 589 (3.82), 645 nm (3.66).

1.1.3. Fmoc-Lys(PS)-OH: 2-(9H-fluoren-9-ylmethoxycarbonylamino)-6-[5-(4-carboxyphenyl)-10,15,20- triphenylporphyrin]-hexanoic acid



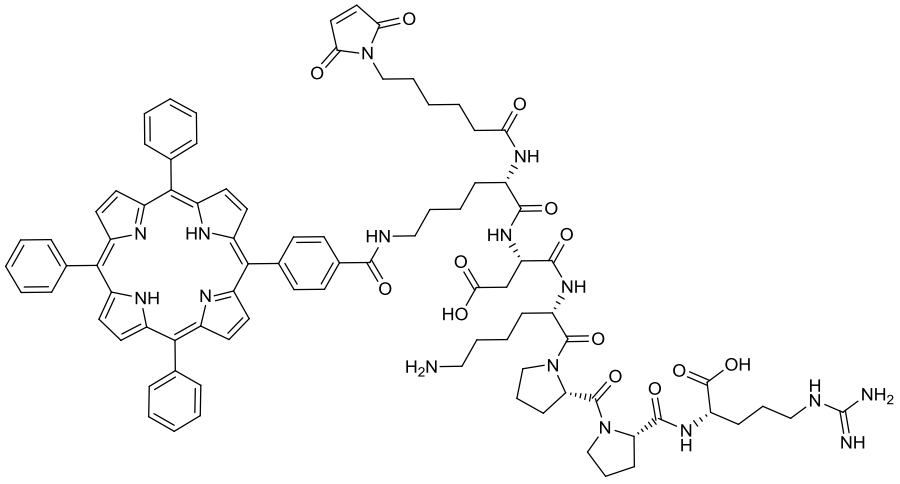
R_f = 0.45 (CH_2Cl_2/EtOH = 95:5, v/v). ^{1}H NMR (DMSO-d_6, 300MHz, δ): -2.9 (s, 2H, NH-pyrrole), 1.70 (m, 6H, $H_{CH2\ \alpha,\beta,\delta}$), 3.44 (s, 2H, $H_{CH2\varepsilon\text{-Lysine}}$), 4.02 (m, 1H, $H_{CH\text{-Fmoc}}$), 4.29 (m, 3H, $2H_{CH2\text{-Fmoc}}$ + $1H_{CH\alpha\text{-Fmoc}}$), 5.75 (s, 1H, NH), 7.71 and 7.33 (2 x m, 8H, $H_{phényl\text{-Fmoc}}$), 7.82 (s, 9H, $H_{p\text{-m phenyl-COOH}}$), 8.24 (d + s, 10H, $6H_o$ + $4H_{o\text{-m phenyl-COOH}}$), 8.82 (d, 8H, $H_{\beta\text{-pyrrole}}$), 10.52 (s, 1H, NH-COOH), 12.59 (br, 1H, OH). HRMS (ESI+): *m/z* calcd. for $C_{66}H_{52}N_6O_5$ $[M + H]^+$, 1009.3999; found 1009.4051. UV/Vis (DMSO): λ_{max}(log ε) = 419 (5.54), 512 (4.11), 546 (3.73), 588 (3.50), 645 nm (3.43).

1.1.4. H-K(PS)DKPPR-OH: PS@KDKPPR



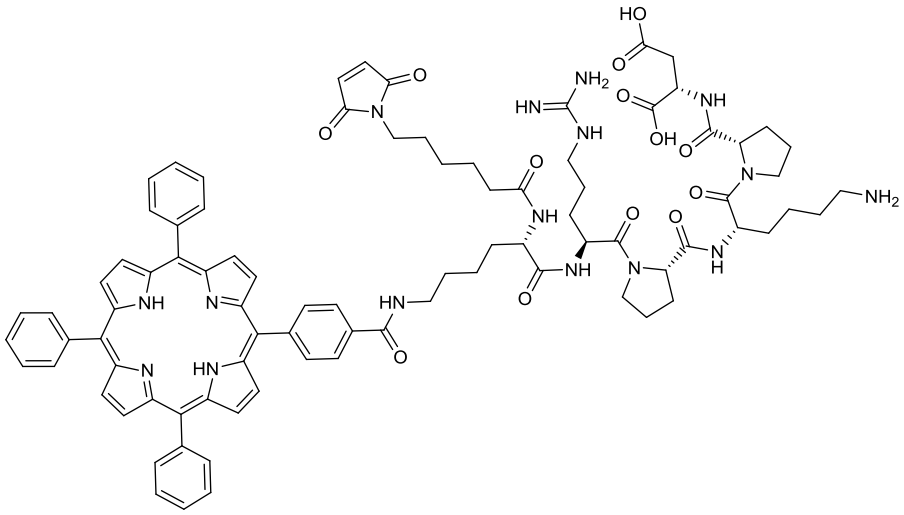
^{1}H NMR (DMSO-d$_6$, 300 MHz, δ): -2.91 (2H, NH-pyrrole), 1.30-1.90 (12H, γCH$_2$ + δCH$_2$ + 2 βH, Lys1 and Lys2), 1.40-1.75 (4H, γCH$_2$ + 2 βH, Arg), 1.83 (3H, γCH$_2$ + βH, Pro), 2.05 (1H, βH, Pro), 2.55, 2.70 (2H, 2 βH, Asp), 2.80 (2H, εCH$_2$, Lys2), 3.10 (2H, δCH$_2$, Arg), 3.32 (2H, εCH$_2$, Lys1), 3.68 (2H, 2 δH, Pro), 4.12 (1H, αH, Arg), 4.29 (1H, αH, Lys1), 4.48 (1H, αH, Lys2), 4.48 (1H, αH, Pro), 4.65 (1H, αH, Asp), 7.60 (1H, εNH, Arg), 7.70 (1H, *ε*NH, Lys2), 7.84 (s, 9H, H$_{p\text{-}m\ phenyl\text{-}COOH}$), 7.95 (1H, NH, Arg), 8.05 (1H, NH, Lys2), 8.18 (1H, *ε*NH, Lys1), 8.22-8.31 (d + s, 10H, 6H$_o$ + 4H$_{o\text{-}m\ phenyl\text{-}COOH}$), 8.75 (1H, NH, Asp), 8.76 (9H, H$_{β\text{-}pyrrole}$, NH, Lys2). HRMS (ESI): *m/z* calcd for C$_{77}$H$_{85}$N$_{15}$O$_{10}$ [M + 2H]$^{2+}$, 690.8375; found, 690.8376 and [M + 3H]$^{3+}$, 460.8941; found, 460.8945. UV/Vis (DMSO): λ$_{max}$(log *ε*) = 419 (5.53), 512 (4.12), 546 (3.77), 588 (3.57), 645 nm (3.50).

1.1.5. Synthesis of Maleimido-K(PS)DKPPR-OH: maleimido-PS@KDKPPR



^{1}H NMR (DMSO-d_6, 300 MHz, δ): -2.92 (2H, NH-pyrrole), 1.21-1.83 (14H, γCH_2 + δCH_2 + 2 βH, Lys1, Lys2 and CH_2 maleimide), 1.40-1.75 (4H, γCH_2 + 2 βH, Arg), 1.83 (3H, γCH_2 + βH, Pro), 2.05 (1H, βH, Pro), 2.15 (4H, CH_2 maleimide), 2.55, 2.70 (2H, 2 βH, Asp), 2.80 (2H, εCH_2, Lys2), 3.10 (2H, δCH_2, Arg), 3.32 (2H, εCH_2, Lys1), 3.41 (2H, CH_2 maleimide), 3.68 (2H, 2 δH, Pro), 4.04 (2H, CH_2 maleimide), 4.12 (1H, αH, Arg), 4.29 (1H, αH, Lys1), 4.48 (1H, αH, Lys2), 4.48 (1H, αH, Pro), 4.65 (1H, αH, Asp), 6.88 (2H, maleimide),7.47 (1H, εNH, Arg), 7.64 (1H, εNH, Lys2), 7.94 (s, 9H, $H_{p\text{-m phenyl-COOH}}$), 7.97 (1H, NH, Arg), 8.01 (1H, NH, Lys2), 8.08 (1H, εNH, Lys1), 8.21 (1H, NH, Asp), 8.22-8.30 (d + s, 10H, $6H_o$ + $4H_{o\text{-m phenyl-COOH}}$), 8.84 (8H, $H_{\beta\text{-pyrrole}}$). HRMS (ESI): *m/z* calcd for $C_{87}H_{96}N_{16}O_{13}$ $[M + 2H]^{2+}$, 787.365; found, 787.8907 and $[M + 3H]^{3+}$, 525.2433; found, 525.2603. UV/Vis (DMSO): $\lambda_{max}(\log \varepsilon)$ = 419 (5.52), 512 (4.08), 546 (3.70), 588 (3.45), 645 nm (3.34).

1.1.6. Maleimido-K(PS)RPKPD-OH : maleimido-PS@scramble



The same protocol was applied for the synthesis of maleimido-K(PS)RPKPD-OH than for the synthesis of maleimido-K(PS)DKPPR-OH, using a Fmoc-Asp(tBu)-Wang resin on a 50 µmole scale. The crude product was further purified by preparative HPLC using acetonitrile/water (0.1 % TFA) [10:90] to 100 % acetonitrile gradient in 15 minutes, followed by isocratic acetonitrile for 10 minutes. R_t = 17.8 min. Pure product was isolated as a green powder (18 mg, 23 %).

^{1}H NMR (DMSO-d_6, 300 MHz, δ): -2.92 (2H, NH-pyrrole), 1.14-1.82 (14H, γCH_2 + δCH_2 + 2 βH, Lys1, Lys2 and CH_2 maleimide), 1.45-1.66 (4H, γCH_2 + 2 βH, Arg), 1.83 (3H, γCH_2 + βH, Pro), 2.07 (1H, βH, Pro), 2.19 (4H, CH_2 maleimide), 2.55, 2.70 (2H, 2 βH, Asp), 2.83 (2H, εCH_2, Lys2), 3.14 (2H, δCH_2, Arg), 3.32 (2H, εCH_2, Lys1), 3.41 (2H, CH_2 maleimide), 3.65 (2H, 2 δH, Pro), 4.04 (2H, CH_2 maleimide), 4.18 (1H, αH, Arg), 4.31 (1H, αH, Lys1), 4.43 (1H, αH, Lys2), 4.50 (1H, αH, Pro), 4.67 (1H, αH, Asp), 6.89 (2H, maleimide),7.43 (1H, εNH, Arg), 7.64 (1H, εNH, Lys2), 7.96 (s, 9H, $H_{p\text{-}m\ phenyl\text{-}COOH}$), 7.98 (1H, NH, Arg), 8.01 (1H, NH, Lys2), 8.07 (1H, εNH, Lys1), 8.21 (1H, NH, Asp), 8.22-8.33 (d + s, 10H, $6H_o$ + $4H_{o\text{-}m\ phenyl\text{-}COOH}$), 8.84 (8H, $H_{\beta\text{-}pyrrole}$). HRMS (ESI): *m/z* calcd for $C_{87}H_{96}N_{16}O_{13}$ $[M + 2H]^{2+}$, 787.365; found, 787.7523 and $[M + 3H]^{3+}$, 525.2433; found, 525.5548. UV/Vis (DMSO): $\lambda_{max}(\log \varepsilon)$ = 419 (5.32), 512 (3.98), 546 (3.65), 588 (3.46), 645 nm (3.40).

1.2. Nanoparticles characterizations

1.2.1. ICP determination of Gd^{3+}

The determination of the accurate concentration of gadolinium in the different samples was performed by ICP-OES (Inductivey Coupled Plasma- Optical Emission Spectroscopy) with a Varian® 710-ES spectrometer. First, particles were degraded overnight in 5 mL of aqua regale (HNO_3 67% mixed with HCl 37% (1:2; v/v) at 80 °C and at an estimated concentration in gadolinium around 0.01 mM, 0.02 mM or 0.05 mM. Subsequently, the samples were diluted to 50 mL with 0.5 M HNO_3 (1:2500, v/v). For the calibration of the ICP-OES, single element standard solution was used and prepared from 1000 ppm Gd-standard from SCP Science® by successive dilutions with an HNO_3 5 % (w/w) matrix. For each particle, the Gd^{3+} composition given is an average of the three samples prepared (at 0.01 mM, 0.02 mM and 0.05 mM).

1.2.2. Relaxivity measurements

Relaxation time measurements were performed using a Bruker Minispec MQ60 NMR analyser, operating at a magnetic field of 1.4 T and at 37 °C. Before measurements of T_1 (longitudinal relaxation time) and T_2 (transverse relaxation time), lyophilized particles were dispersed in water for one hour at room temperature, $[Gd^{3+}]$ = 100 mM and pH=7,4. For AGuIX, r_1 = 12.4 $s^{-1}.mM^{-1}$ per Gd^{3+} and r_2 = 17.4 $s^{-1}.mM^{-1}$ per Gd^{3+} (r_2/r_1 = 1.4). For AGuIX@PS, r_1 = 17.6 $s^{-1}.mM^{-1}$ per Gd^{3+} and r_2 = 28.4 $s^{-1}.mM^{-1}$ per Gd^{3+} (r_2/r_1 = 1.6). For AGuIX@SH, r_1 = 14.5 $s^{-1}.mM^{-1}$ per Gd^{3+} and r_2 = 23.2 $s^{-1}.mM^{-1}$ per Gd^{3+} (r_2/r_1 = 1.6). For AGuIX@PS@KDKPPR, r_1 = 16.1 $s^{-1}.mM^{-1}$ per Gd^{3+} and r_2 = 25.8 $s^{-1}.mM^{-1}$ per Gd^{3+} (r_2/r_1 = 1.6). For AGuIX@PS@scramble, r_1 = 15.4 $s^{-1}.mM^{-1}$ per Gd^{3+} and r_2 = 24.6 $s^{-1}.mM^{-1}$ per Gd^{3+} (r_2/r_1 = 1.6).

1.2.3. Dynamic Light Scaterring size measurement

Direct measurements of the hydrodynamic diameter distribution of the nanoparticles were performed *via* a Zetasizer NanoS DLS (laser He-Ne 633 nm) from Malvern Instruments. For measurements, lyophilized particles were first dispersed in water for one hour at room temperature, $[Gd^{3+}]$ = 100 mM and pH=7.4. Then particles were diluted to $[Gd^{3+}]$ = 2.5 mM and measurements immediately taken. The average hydrodynamic diameter (in number) measured by DLS was 3.3 nm (standard deviation: 0.8) for AGuIX, 8.0 nm for AGuIX@PS standard deviation: 1.4), 4.7 nm for AGuIX@SH (standard deviation: 1.2), 9.0 nm for AGuIX@PS@KDKPPR (standard deviation: 2.3) and 10.6 nm for AGuIX@PS@scramble (standard deviation: 2.3).

1.2.4. Porphyrin quantification on nanoparticles by UV-visible measurements

Solution of the maleimido-PS@KDKPPR compound was prepared at 1 mM in DMSO and diluted in water for analysis by UV−Vis spectroscopy in the range of 200 to 800 nm. An UV−Vis spectrophotometer (Varian Cary50) and a Hellma semimicro cell, 1 cm light path, 1400 µL, manufactured from Suprasil quartz were used. Calibration curves were therefore obtained for Q bands

at 520 nm, 555 nm, 590 nm and 650 nm (0 – 50 µM) and for the Soret Band at 420 nm (0 – 2.5 µM). To quantify the number of maleimido-PS@KDKPPR compound per particle, AGuIX@PS@KDKPPR solution were diluted in water and absorbance were measured at 520 nm, 555 nm, 590 nm, 650 nm and 420 nm. The average result gives 1 PS@KDKPPR for 15 Gd^{3+} (1 PS@KDKPPR for 1-2 particles).

The same procedure is followed for the quantification of maleimido-PS@scramble compound on AGuIX@PS@scramble. The average result gives 1 PS@scramble for 13 Gd^{3+} (1 PS@KDKPPR for 1-2 particles).

2. Supplemental tables and figures

Figure S1. Photophysical properties of PS@KDKPPR and AGuIX@PS@KDKPPR in water. **S1.a)** Absorbance spectra recorded with an UV−Vis spectrophotometer (Varian Cary50) and a Hellma semimicro cell, 1 cm light path, 1400 µL, manufactured from Suprasil quartz. No change in the absorbance spectra is detected after grafting of the maleimido-PS@KDKPPR on particle. **S1.b)** Fluorescence spectra recorded with a Varian Cary Eclipse fluorescence spectrophotometer and a Hellma semimicro cell, 1400 µL, manufactured from Suprasil quartz. Excitation wavelength: 420 nm, Emission and excitation slits: 5 nm, data interval: 1 nm, averaging time: 0.1 s, range: 600-800 nm.

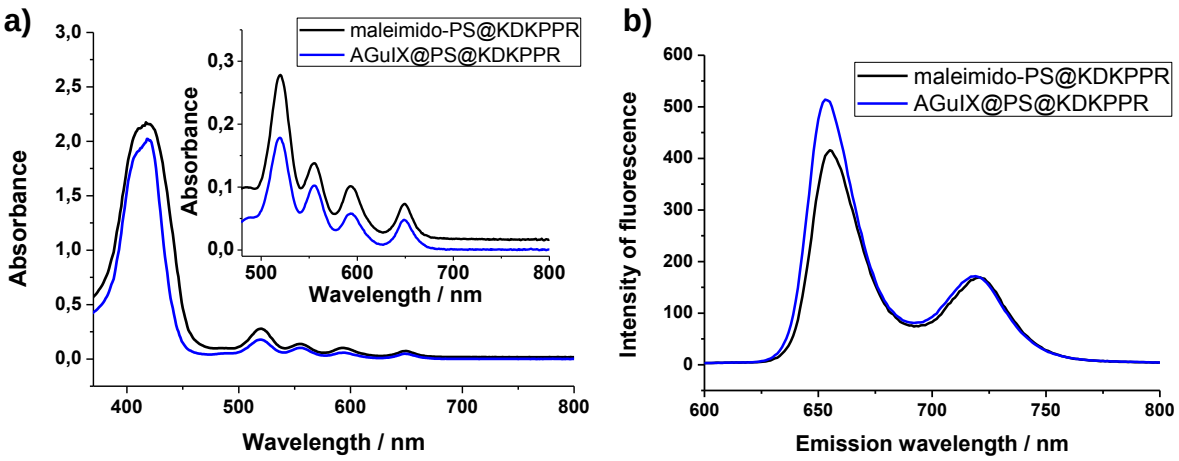


Table S1. Colloidal stabilidy of the AGuIX@PS@peptide nanoparticles. A solution of AGuIX@PS@peptide nanoparticles in water ($[Gd^{3+}]$ = 50 mM) was diluted in human serum (AB) to reach a concentration $[Gd^{3+}]$ ~ 5 mM**.** The longitudinal and transversal relaxation times and the size, were immediately recorded as described previously. The solution was then kept at 37 °C and measurement performed 1 h, 5 h, and 24 h after dilution in the serum.

Even after 24 h, the size is not significantly modified. No increase of the size indicates that there is no agglomeration of the nanoparticles and no decrease of the size indicates that there is no dissolution of the polysiloxane matrix. Colloidal stability is maintained. Besides, relaxometric measurements indicate that the structure of the particles is preserved since the relaxivities r_2 and r_1 are almost identical. Relaxivities are directly correlated with the size of the nanoparticle and the absence of variation is correlated with the stability of the nanoparticles in the medium. For comparison, if the nanoparticles were completely degraded, the polysiloxane core would be hydrolyzed leading to the formation of free DOTAGA(Gd^{3+}) complexes and to a r_1 around 4 $s^{-1}.mM^{-1}$.

	0 h (dilution in the serum)	1 h	5 h	24 h
r_1 ($s^{-1}.ms^{-1}$)	17.7	17.2	16.1	14.4
r_2 / r_1	1.7	1.7	1.7	1.8
Size (nm)	10.3 ± 2.8	11.9 ± 2.7	11.3 ± 2.4	11.9 ± 2.8

Figure S2 *In vitro* dark cytotoxicity. Cell survival after incubation with nanoparticles in the dark was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. HUVEC were plated at an initial cell density of 5×10^4 cells/well in 24-well plates and allowed to attach overnight. Wells were rinsed twice with PBS and filled with 500 µL HUVEC medium containing various concentrations of nanoparticles (0.1 or 10 µM). After a 24-h incubation at 37 °C, wells were rinsed twice with cold PBS and filled with 200 µL HUVEC medium. Cell survival was measured by MTT assay as previously described (3). Experiments were carried out in triplicates. The results indicates that nanoparticles yielded no cytotoxicity.

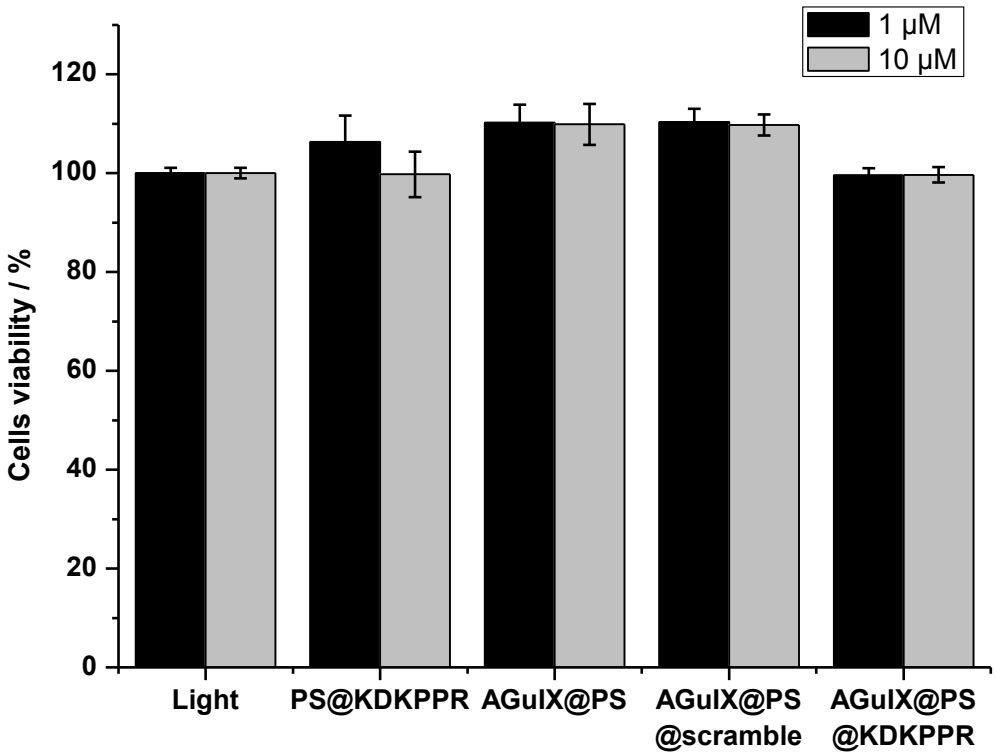
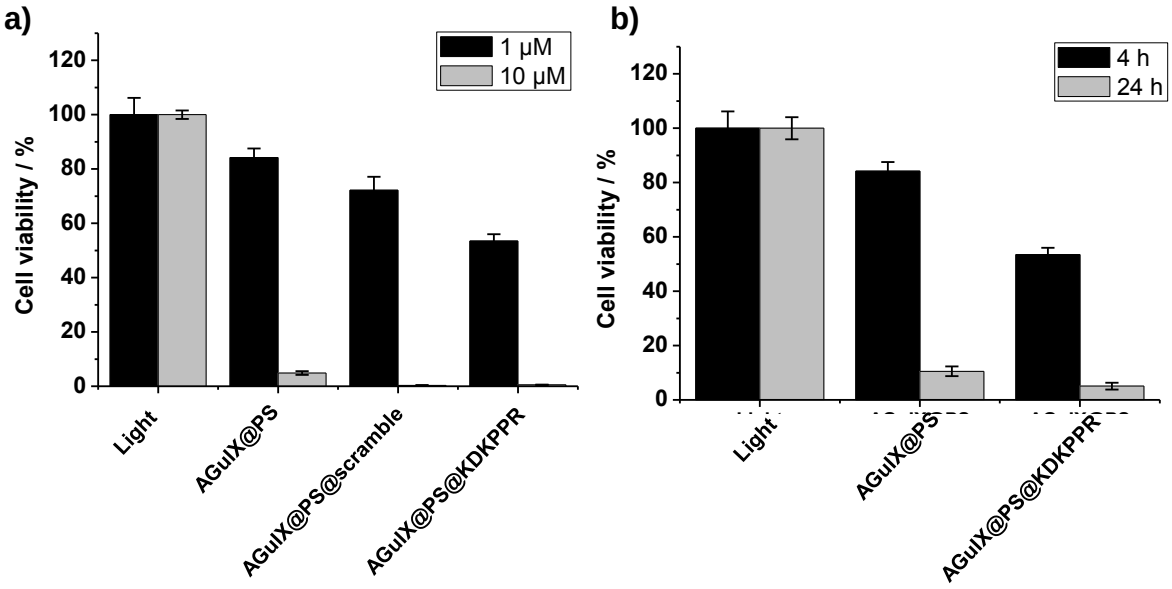


Figure S3. *In vitro* photocytotoxic effect of functionalized particles on HUVEC cells. **S3.a)** Effect of concentration: cell viability after PDT (5 J.cm^{-2}, 4 h exposition) following the exposition with particles at a concentration of 1 or 10 µM (concentration indicated in PS). **S3.b)** Effect of contact time: cell viability after PDT (5 J.cm^{-2}, [PS] = 1 µM) following the exposition during 4 or 24 h with particles. Control correspond to cells irradiated in absence of nanoparticles.



3. References

1. Seybold PG, Gouterman M. 1969. Porphyrins. J Mol Spectrosc 31:1–13.

2. Redmond RW, Gamlin JN. 1999. A Compilation of Singlet Oxygen Yields from Biologically Relevant Molecules. Photochem Photobiol 70:391–475.

3. Benachour H, Sève A, Bastogne T, Frochot C, Vanderesse R, Jasniewski J, Miladi I, Billotey C, Tillement O, Lux F, Barberi-Heyob M. 2012. Multifunctional Peptide-Conjugated Hybrid Silica Nanoparticles for Photodynamic Therapy and MRI. Theranostics 2:889–904.