Supplementary Materials

Smart Dual-Functionalized Gold Nanoclusters for Spatio-Temporally Controlled Delivery of Combined Chemo- and Photodynamic Therapy

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1. Synthesis of undecyl-tetraethyleneglycol ligands

The procedure for the synthesis of the ligands was adapted from those described in the literature (Scheme S1).1,2 All spectral data is consistent with those previously published. Specific synthetic procedures are detailed below. Tetrachloroauric (III) acid (HAuCl4.xH2O), tetraethyleneglycol, 11-bromo-1-undecene, thioacetic acid, irgacure-651® (2,2-Dimethoxy-1,2-diphenylethan-1-one), sodium borohydride, and dialysis sacks (MWCO 12,000 Da) were purchased from Sigma-Aldrich® and used as received.



**Scheme S1 Synthetic route for the preparation of 23-mercapto-3,6,9,12-tetraoxatricosan-1-ol (4).**

**Synthesis of 3,6,9,12-tetraoxatricos-22-en-1-ol:** A mixture of tetraethyleneglycol (35 mL, 202 mmol, 15 eq) and NaOH (900 mg, 22.5 mmol, 1.2 eq) were refluxed for 30 minutes. 11-Bromoundecene (4.5 g, 19 mmol) was then added and the reaction mixture was refluxed overnight. 50 mL of water were then added and the product was extracted 5 times with diethyl ether. After evaporation, the colorless oil was purified by silica column chromatography eluted with ethyl acetate:cyclohexane v = 1:1 (5.3 g, 22.5 mmol, 80%). 1H NMR (400 MHz, CDCl3) δ 5.78 (m, 1H), 4.95 (m, 2H), 3.70 (t, 2H, *J* = 4.5 Hz), 3.55-3.65 (m, 14H), 3.42 (t, 2H, *J* = 6.0 Hz), 2.00 (m, 2H), 1.53 (m, 2H), 1.32 (m, 2H), 1.25 (m, 10H).

**Synthesis of S-(1-hydroxy-3,6,9,12-tetraoxatricosan-23-yl) thioacetate:** Irgacure 651 (30 mg, 0.01 mmol, 0.02 eq) was added to a mixture of compound **2** (950 mg, 2.7 mmol) and thioacetic acid (0,4 mL, 5,5 mmol, 2 eq). The mixture was irradiated for 3h at room temperature in a LuzChem LZC-4 photoreactor (15 W, UVB lamps). Then, diethyl ether was added and the organic layer was washed 3 times with water. The organic layers were evaporated to provide a pale-yellow oil used without further purification (900 mg, 2,2 mmol, 80%). 1H NMR (400 MHz, CDCl3) δ 3.72 (t, 2H, *J* = 4.5 Hz), 3.55-3.65 (m, 14H), 3.44 (t, 2H, *J* = 7.0 Hz), 2.85 (t, 2H, *J* = 7.0 Hz), 2.31 (s, 3H), 1.55 (m, 4H), 1.25 (m, 14H).

**Synthesis of 23-mercapto-3,6,9,12-tetraoxatricosan-1-ol.** A mixture of compound **3** (900 mg, 2.2 mmol) in MeOH (20 mL) and HCl 37% (20 mL) was refluxed for 6h. The reaction was quenched by slow addition of a solution of KOH (1 M). The resulting crude was extracted 5 times with dichloromethane and evaporated under vacuum to provide a colorless oil (650 mg, 80%). 1H NMR (400 MHz, CDCl3) δ 3.71 (t, 2H, *J* = 4.5 Hz), 3.55-3.65 (m, 14H), 3.42 (t, 2H, *J* = 7.0 Hz), 2,51 (q, 2H, *J* = 7.0 Hz), 1.55 (m, 2H), 1.25 (m, 16H).

2. Synthesis of the singlet oxygen-cleavable linker (*Z*)-3,3'-(ethene-1,2-diylbis(sulfanediyl))dipropanoic acid



The procedure for the synthesis of the singlet oxygen-cleavable linker was adapted from that described by Wang et al.3 3-Mercaptopropionic acid (3-MPA) (1.7 mL, 19.5 mmol) was added to a stirred solution of 30 wt % NaOMe in MeOH (7.5 mL, 39.0 mmol) at 0°C. After 3 h of stirring, the uncolored mixture turned to a white suspension. Then, methanol was evaporated, and the resulting white salt was dried in vacuum. To a solution of disodium salt of 3-MPA in dry DMF (15 mL) was added *cis*-1,2-dichloroethylene (1 g, 10.5 mmol) in one portion. The reaction mixture was stirred at 50ºC for 18 h and then cooled to RT. After diluted with water (50 mL), the solution was acidified to pH = 3 with KHSO4 (1 M). The mixture was washed with EtOAc (300 mL) and the combined organic layers were washed with water (200 mL), brine (100 mL), and dried over MgSO4 anhydrous and concentrated under reduced pressure. The product was washed with petroleum ether to obtain brown powders, which were recrystallized from EtOAc/hexane and dried under vacuum to afford the desired diacid as yellowish powders (880 mg, 5.8 mmol, 30%). The physical and spectroscopic data of (Z)-3,3'-(ethene-1,2-diylbis(sulfanediyl))dipropanoic acid were identical to those reported in the literature (Wang *et al*., 2016). 1H NMR (CD3OD): *δ* 6.20 (s, 2H), 2.94 (td, *J* = 7.1, 0.4, 4H), 2.63 (td, *J* = 7.1, 0.4, 4H). 13C NMR (CD3OD): *δ* 175.4 (C), 124.9 (CH), 36.3 (CH2), 29.9 (CH2). MS (EI) *m/z* (rel intensity) C8H12O4S2 236.1 (100).

3. Synthesis of gold nanoclusters

34 mg of 23-mercapto-3,6,9,12-tetraoxatricosan-1-ol (100 μmol) were mixed with 10 mg of tetrachloroauric acid trihydrate (30 μmol) in 7.0 mL of MeOH. 0.6 mL of a freshly prepared solution of sodium borohydride (1.3 M) was added at an input rate of 4.8 mL/h under vigorous stirring. The mixture was stirred at room temperature for 3h and the solvent was removed under reduced pressure. The as-prepared nanoclusters were dispersed in 15 mL of water and extensively purified by dialysis. The resulting structure was **Au10(SR)152-** with chemical composition Au10C285H585O75S15 (see characterization below).

Nuclear magnetic resonance (NMR) spectroscopy was first used to assess the formation of gold nanoclusters. 1H-NMR spectra were recorded on a Varian 400-MR spectrometer working at 400 MHz. Samples were dissolved in either CDCl3 or D2O. Chemical shifts are reported in parts per million (ppm, δ) and are referenced to the residual signal of the solvent. Coupling constants are reported in Hertz (Hz). Spectral splitting patterns are designated as s (singlet), d (doublet), t (triplet), q (quartet), m (complex multiplet). It is well known that upon formation of thiol protected gold nanoparticles, the NMR spectra of the nanomaterials shows broader signals as compared to those of the ligands in solution. This notion was used to further confirm the formation of AuNCs for the sample bearing –OH terminated tetraethyleneglycol undecyl-thiol ligands (Fig. S1).



**Figure S1.** NMR spectra of –OH terminated tetraethyleneglycol undecyl-thiol ligands (red) and AuNCs (blue).

Electrospray mass ionization (ESI) spectra of tetraethyleneglycol alkyl-thiol stabilized gold nanoclusters were acquired in an LTQ FT ultra mass spectrometer (Thermo Fisher Scientific, detection limit 4000 Da). The purified sample was injected using a syringe pump with a 10 µL.min-1 flow rate and each spectrum was collected in negative mode (capillary and tube lens voltages of -45 and -90 V, respectively). The mass spectrum of the AuNCs shows major peaks centered at m/z 3830.5, 3451.3, and 3071.97 (Fig. S2).



**Figure S2.** ESI mass spectrum of AuNCs. .

To assign a molecular formula for the clusters, a series of fragmentations simulations were performed varying the number of ligands and gold atoms for the nanoclusters. Best fittings were found when the structure **Au10(SR)152-** was simulated, which has a predicted m/z of 3830.72 while the found molecular ion is 3830.4830 (Figure S3). Moreover, in this case, the following two most abundant fragmentations could be assigned to the loss of two thiol ligands (Fig. S3).



**Figure S3.** (**A**) Experimental mass spectrum of gold nanoclusters; (**B**) theoretical mass spectrum corresponding to Au10(SR)152- nanocluster, with chemical composition Au10C285H585O75S15; (**C**) theoretical mass spectrum corresponding to Au10(SR)132- nanocluster, with chemical composition Au10C247H507O65S13 and (**D**) theoretical mass spectrum corresponding to Au10(SR)112- nanocluster, with chemical composition Au10C209H429O55S11.

Additional structural characterization was obtained by transmission electronic microscopy (TEM) images and zeta potential measurements. TEM images were collected using a JEOL JEM-1400PLUS TEM operated at an accelerating voltage of 120 kV. The cluster solutions were drop-casted onto a 3-4 nm thick film of amorphous carbon supported on a 400 mesh copper grid (Ted Pella Inc., no. 01822-F). Then the sample was dried at room temperature under ambient condition. The microscope magnification was calibrated with a calibration grid (Grating Replica, Waffle, 2160 l/mm, on a 3 mm grid, Ted Pella Inc., no. 607). The TEM examination revealed the formation of gold nanoclusters, mainly spherical and of 1.5 nm of average size, evaluated from several micrographs (Fig. 1 in the main text). Zeta potential of aqueous solutions of gold nanoclusters was determined using a Malvern® Zetasizer Nano-ZS equipment. In aqueous solution, the zeta potential was -2 mV.

4. Conjugation of Protoporphyrin IX and Doxorubicin to the gold nanoclusters

**Synthesis of AuNCs-PpIX**. 60 mg of AuNCs were mixed with 8 mg of PpIX, 75 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and 2 mg of dimethylaminopyridine (DMAP) in 12 mL of anhydrous tetrahydrofuran. The mixture was stirred for 12 h and the solvent was removed under reduced pressure. The as-prepared nanoclusters were dispersed in 15 mL of dimethylsulfoxide (DMSO) and extensively purified by water dialysis.

**Synthesis of AuNCs-DOX**. 85 mg of (*Z*)-3,3’-(ethene-1,2-diylbis(sulfanediyl))dipropanoic acid, 55 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and 9 mg of dimethylaminopyridine (DMAP) were mixed in 10 mL of anhydrous THF and stirred for 30 min. Then, 40 mg of AuNCs in 10 mL of anhydrous THF was added and the mixture was stirred for 24 h. The solvent was removed under reduced pressure and the clusters were redispersed in 30 mL of water. Finally, the clusters were extensively purified by water dialysis.

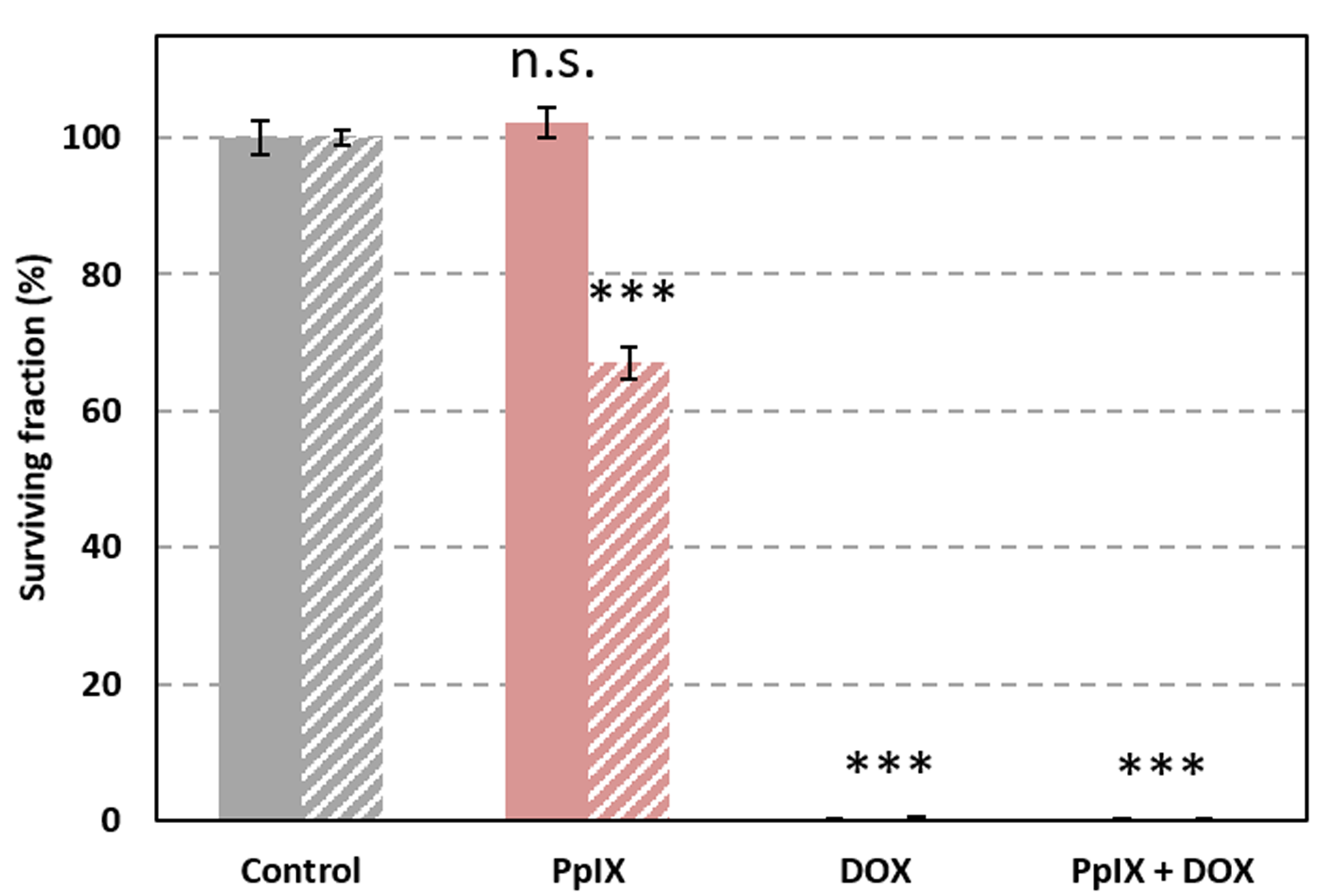
65 mg of the as-prepared AuNCs with a 1O2 sensitive linker was mixed with 20 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), 15 mg of *N*-hydroxysuccinimide in 20 mL of DMSO. After 30 min of vigorous stirring, 5 mg of doxorubicin and 2 mg of dimethylaminopyridine (DMAP) were added to the mixture and it was stirred for 24 h in the dark. Finally, the clusters were extensively purified by water dialysis.

**Synthesis of AuNCs-PpIX-DOX** 40 mg of AuNCs-PpIX was mixed with 50 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 50 mg of *N*-hydroxysuccinimide in 20 mL of DMSO. After 30 min of vigorous stirring, 50 mg of the 1O2-linker, and 2 mg of dimethylaminopyridine (DMAP) were added to the mixture and it was stirred for 24 h in the dark. Finally, the clusters were extensively purified by water dialysis.

The as-prepared clusters were subsequently mixed with 40 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), 30 mg of *N*-hydroxysuccinimide in 20 mL of DMSO. After 30 min of vigorous stirring, 5 mg of doxorubicin and 2 mg of dimethylaminopyridine (DMAP) were added to the mixture and it was stirred for 24 h in the dark. Finally, the clusters were extensively purified by water dialysis.

5. Individual and combined treatment with free Protoporphyrin IX and Doxorubicin in HeLa cells

Subconfluent cells seeded in F25 flasks or 24-well plates were incubated for 18 h with different treatments (PpIX 2.5 µM, Dox 1.5 µM or Combined treatment of PpIX 2.5 µM + Dox 1.5 µM), washed three times with culture medium without FBS and maintained in the culture medium during irradiation and post-treatment time. Irradiations were performed with a red (λ max = 632 nm) light-emitting diode (LED) device (LED Par 64 Short; Showtec, Burgebrach, Holland), using a total light dosage of 2.4 J·cm-2 (total light dose = fluence rate x treatment time). Fluence rate [mW·cm-2] was measured with the PM100A handheld optical power meter (Thorlabs, Newton, New Jersey, USA). Control experiments were carried out by incubating the same compound without irradiation, to examine the possible cytotoxic effect (dark cytotoxicity) exerted by compounds.



**Figure S4.** MTT viability assay 48 h after individual and combined treatments with free drugs in dark conditions (plain bars) and after 2.4 J/cm2 of red light (striped bars). Data correspond to mean ± S.D. values from at least six different experiments. Statistically-significant differences are labeled as \*\*\*p<0.001, n.s. non-significant.

6. Time-lapse video microscopy of HeLa cells after Combined Bimodal treatment

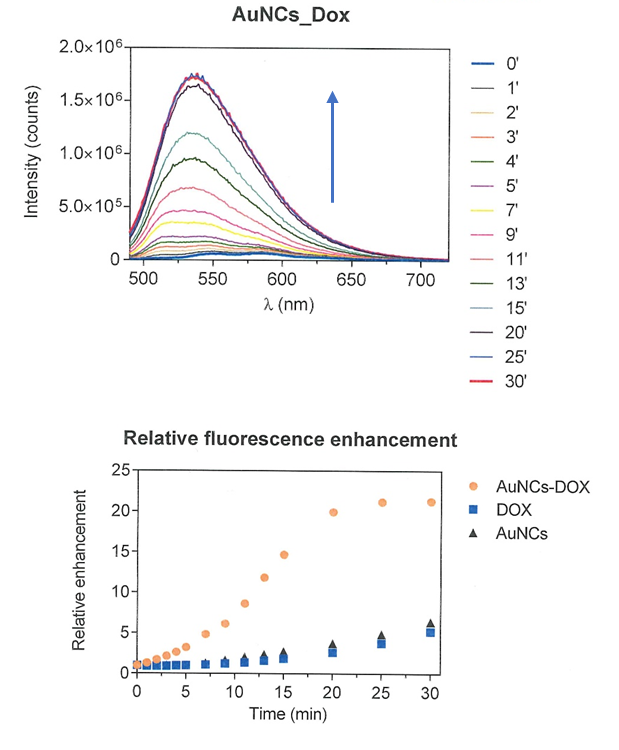
Just after irradiation of control and combined bimodal treated cells, 24 h time-lapse videomicroscopy was carried out with a Leica DMI6000B microscope with an incubation system and OrcaR2 monochrome digital camera for image detection (Hamamatsu Photonics, Shizuoka, Japan).

**Video S1 –** Control HeLa cells.

**Video S2 –** Combined bimodal treated HeLa cells.

7. Singlet oxygen-induced release of Doxorubicinfrom AuNC-DOX nanoconjugates

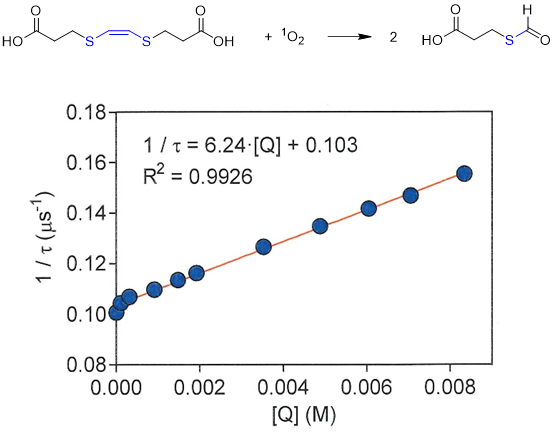
Release of DOX from the nanoconjugates upon reaction of singlet oxygen with the cleavable linker was studied by fluorescence spectroscopy. Thus, to a sample of the AuNC-DOX nanoconjugate in methanol, an aliquot of the singlet oxygen photosensitizer phenalenone-2-sulfonate4 was added and irradiated with blue light using a LED source (LED Par 64 Short; Showtec, Burgebrach, Holland). The fluorescence of DOX was recorded in a Fluoromax 4 spectrofluorometer (Jobin-Yvon Horiba, Japan) at different time intervals and was observed to increase until a plateau value was reached, as shown in Figure **S5**



**Figure S5.** Singlet oxygen-induced release of Doxorubicin from Gold nanocluster-doxorubicin conjugates bound through a singlet oxygen cleavable linker. As controls, the fluorescence of the non-functionalized nanoclusters and of free doxorubicin was recorded under the same conditions.

8. Reactivity of singlet oxygen with the cleavable linker

The reaction of singlet oxygen with the cleavable linker was studied monitoring the rate of decay of the singlet oxygen phosphorescence in methanol solutions containing increasing amounts of the linker. A defined amount of singlet oxygen was generated in the solution by pulsed laser irradiation of the cationic porphyrin meso-tetra(4-N-methylpyridyl)porphine (TMPyP), a well-known singlet oxygen photosensitizer, at 532 nm using using a diode pumped frequency doubled Q-switched Nd:YAG laser (Crylas, Germany). The phosphorescence of singlet oxygen was monitored at 1275 nm using a dedicated photomutiplier (H9170-45, Hamamatsu Photonics, Hamamatsu, Japan) and the rate constant for decay (*k*) *was* determined by exponential fitting of the phosphorescence decay signal. It was found to increase linearly with the concentration of the linker [Q] (Figure **S6**).5



**Figure S6.** Rate constant for the reaction of singlet oxygen with the linker. The rate of singlet oxygen disappearance increased linearly with the concentration of the linker.

Linear regression of the data in Figure S5 yielded the rate constant for reaction between singlet oxygen and the linker, *k*q:



Where *k*0 is the decay rate constant in the absence of linker and [Q] the concentration of the linker. The value found was *k*q = 6.24 x 106 M-1 s-1.

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