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## Photo-activated drug delivery systems

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### Citation

Kong, L. (2018, June 7). *Photo-activated drug delivery systems*. Retrieved from <https://hdl.handle.net/1887/63080>

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**Author:** Kong, L.

**Title:** Photo-activated drug delivery systems

**Issue Date:** 2018-06-07

# 3

## **Spatiotemporal Control of Doxorubicin Delivery from “Stealth-Like” Prodrug Micelles**

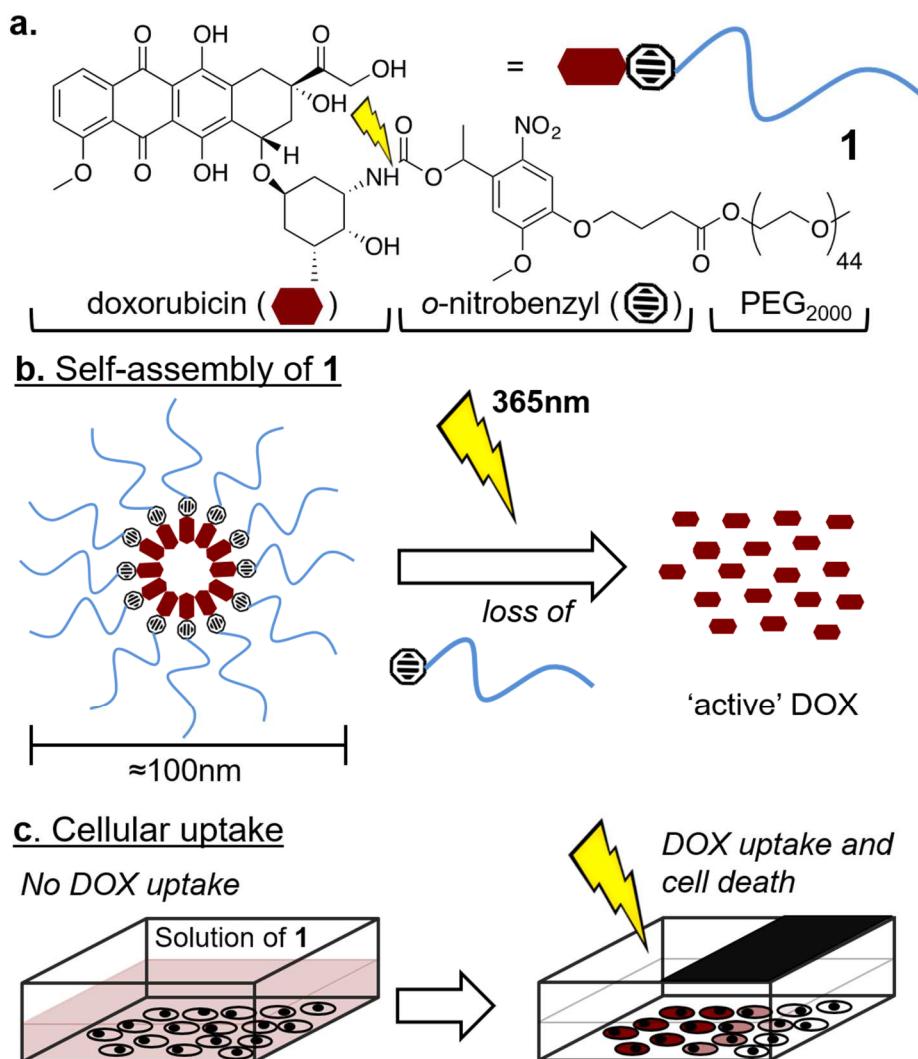
**Abstract:** In the treatment of cancer, targeting of anticancer drugs to the tumor microenvironment is highly desirable. Not only does this imply accurate tumor targeting but also minimal drug release *en route* to the tumor and maximal drug release once there. Here we describe high-loading, “stealth-like” doxorubicin micelles as a pro-drug delivery system, which upon light activation, leads to burst-like doxorubicin release. Through this approach, we show precise spatiotemporal control of doxorubicin delivery to cells *in vitro*.

### 3.1 Introduction

Doxorubicin (DOX) is a potent cytotoxic drug used in the clinical treatment of many human cancers. Administered alone, and with no inherent cell selectivity, the clinical efficacy of DOX is however hampered by off-target cardiotoxicity.<sup>[1]</sup> This limits the cumulative patient lifetime dose of DOX to just 550 mg/m<sup>2</sup>, irrespective of therapeutic success.<sup>[2]</sup> Considerable efforts have been made to improve the therapeutic index of DOX by localizing its extracellular release to the tumor microenvironment alone. Typically, this involves chemical modification or vector entrapment of DOX (*e.g.* within long-circulating liposomes). Within these systems, strategies to enhance tumor targeting and/or local DOX release include the use of active targeting ligands,<sup>[3]</sup> steric shielding (*e.g.* PEGylation) of DOX carriers,<sup>[4]</sup> exploitation of endogenous (*e.g.* low pH within the tumor environment) and exogenous (*e.g.* heat, magnetism, ultrasound or light) stimuli,<sup>[5]</sup> and combinations thereof.<sup>[6]</sup>

Of these various approaches, the passive targeting of liposome-entrapped DOX to tumors remains the only strategy approved for clinical use. Liposomal-DOX formulations (*e.g.* Myocet®, Doxil®) are used to treat a variety of malignant human cancers, including select breast and ovarian cancers, multiple myeloma and AIDS-related Kaposi's sarcoma. These liposome formulations, optimally 100 nm in size and administered systemically, are designed to passively accumulate within the tumor via the enhanced permeability and retention (EPR) effect. This phenomenon is characterized by the ill-defined ("leaky") vasculature and poor lymphatic drainage of many tumor pathologies.<sup>[7]</sup> Through this approach high local concentrations of DOX are achieved within the tumor following prolonged and passive drug leakage across the liposome membrane. For this strategy to be effective, liposomes with long circulation lifetimes are sought. Care must therefore be taken to balance the conflicting need to both minimize DOX leakage *en route* to the tumor while ensuring therapeutically relevant concentrations are released once there. Drug retention and release profiles can be fine-tuned through judicious choice of drug-to-lipid ratios and liposome lipid composition, and circulation lifetimes can be increased through steric shielding (typically PEGylation) of the liposome surface (to create 'stealth' liposomes)

<sup>[4]</sup>, however finding the necessary balance between drug retention and release is an intrinsic limitation of these nanoparticle systems.<sup>[8]</sup>



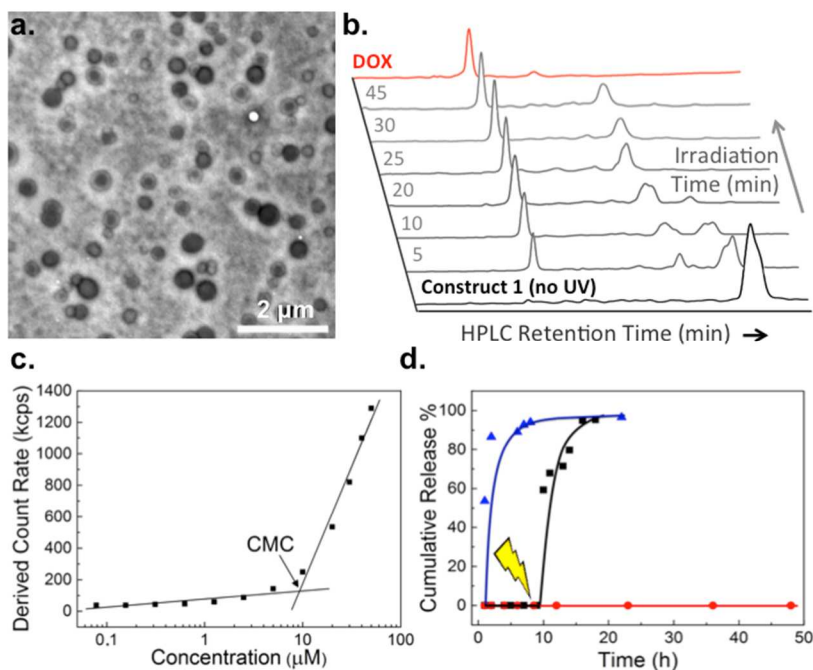
**Figure 1.** Light activated doxorubicin pro-drug micelles. (a) Doxorubicin-ortho-nitrobenzyl-mPEG2000 construct, **1**; (b) Self-assembly of **1** in aqueous media to 100 nm PEGylated and DOX (doxorubicin)-rich micelles from which quantitative drug release is triggered by light; (c) Light directed DOX release, cell uptake, and cell death.

In this chapter, we describe light activated, DOX-rich (20 wt% drug loading) micelles, which prior to light activation, share analogous physicochemical properties (size, morphology, surface chemistry) to those of long circulating liposomal-DOX

formulations. Crucially however we observe no premature DOX release (and therefore cytotoxicity) in the absence of light. Upon light activation, quantitative drug release is achieved (Figure 1). These properties represent a significant technological improvement over analogous DOX-PEG prodrug systems triggered by tumor-specific, endogenous stimuli (pH,<sup>[9]</sup> reduction,<sup>[10]</sup> enzymatic<sup>[11]</sup>), for which DOX release is typically slow (hours) and incomplete, as well as those reliant on external stimulus, such as light,<sup>[12]</sup> for which reported physicochemical properties (size, morphology, surface chemistry) preclude long circulation lifetimes necessary for efficient tumor accumulation via the EPR effect.

### 3.2 Results and discussion

The synthesis and characterisation of photoactivatable DOX-ortho-nitrobenzyl-PEG construct, **1**, is described in the experimental section. Self-assembly of **1** in aqueous media resulted in particles with mean hydrodynamic diameters of 100 nm and ranging in size from 30 to 300 nm (PDI 0.25, Figure S7). TEM (transmission electron microscopy) measurements revealed “loose” core-shell micelle structures in which the nanoparticle core appears electron-rich (high contrast) and likely contains DOX (Figure 2a). Similar morphologies have been reported for analogous DOX-PEG assemblies.<sup>[9]</sup> The critical micelle concentration (CMC) of self-assembled micelles of **1** was determined to be 9.2  $\mu\text{M}$  (approx. 25  $\mu\text{g/mL}$ , Figure 2b) and particles were stable over time, over a range of concentrations and diluted in complete cell culture media (Figure S7). Upon low power UV irradiation (365 nm, 3–5  $\text{mW/cm}^2$ ), complete photolysis of self-assembled **1** to pharmacologically “active” DOX was achieved within 25 min, however significant DOX release was observed following just 5 min low-power UV irradiation (Figure 2c). Drug release was quantitative and importantly, no premature leakage of DOX was observed in the absence of light activation (Figure 2d).

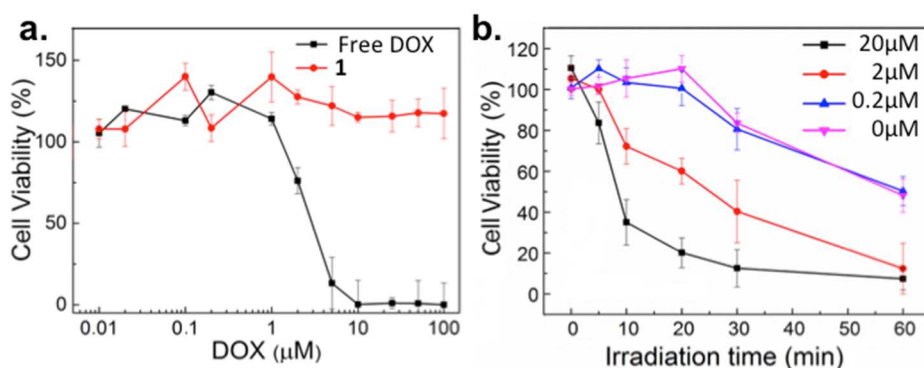


**Figure 2.** Characterization of doxorubicin pro-drug micelles and light induced drug release. (a) TEM image (uranyl acetate stain) of micelles of **1** (300 μM, approx. 0.7 mg/mL); (b) Time evolution of the HPLC spectra of a solution of **1** (100 μM in PBS) during photolysis (365 nm, 3–5 mW/cm<sup>2</sup>). Free DOX (100 μM), dissolved in PBS, was used to confirm clean photolysis of **1** to release “active” DOX. HPLC conditions described in Materials and Methods; (c) CMC (critical micelle concentration) determination by light scattering following serial dilution of **1** (100 μM–75 nM) in PBS; (d) *In vitro* DOX release profiles from **1** (300 μM) in PBS. No UV irradiation (**red**), UV irradiation at 9 h (**black**) and free DOX control (**blue**).

Next, the cytotoxicity of **1** was assessed against cancer (HeLa) cells *in vitro*. While the measured IC<sub>50</sub> value of free DOX was 3 μM, **1** showed no cytotoxic effect up to the highest concentration tested (100 μM) in the absence of light (Figure 3a). Upon light activation (365 nm, 15–17 mW/cm<sup>2</sup>) however, DOX induced cytotoxicity correlated, as expected, with both increased concentrations of **1** as well as increasing irradiation time (Figure 3b).

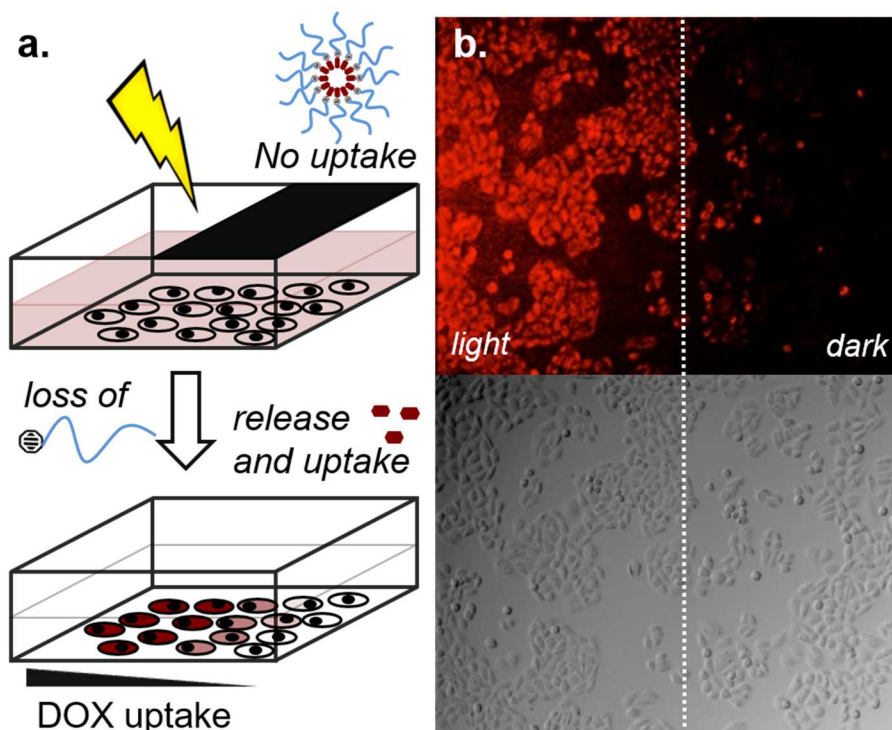
Importantly, UV-A light induced cytotoxicity (due to UV-A induced oxidative stress),<sup>[13]</sup> only resulted in significant cell death following > 30 min continuous irradiation (Figure 3b, pink line, and Figure S8). This is significantly longer than the irradiation time

required to release effective concentrations of DOX (released from 20  $\mu\text{M}$  solutions of **1**) achieving > 50% cell death. It is also important to note, below its CMC (9.2  $\mu\text{M}$ ), the cytotoxicity of **1** was also insignificant. While this is likely due to the membrane impermeability of individual DOX-PEG constructs, these systems will no longer exist as nanoparticle assemblies and will likely demonstrate very different *in vivo* pharmacokinetic profiles (*i.e.* low vascular retention, rapid renal filtration) compared to 100 nm micelles of **1**.<sup>[14]</sup>



**Figure 3.** Viability of HeLa cells *in vitro* treated with doxorubicin pro-drug micelles. (a) Cell viability following incubation with varying concentrations (10 nM–100  $\mu\text{M}$ ) of free DOX (**black**) and **1** (**red**) in the absence of light; (b) Viability of HeLa cells *in vitro* treated with varying concentrations of **1** and irradiated (365 nm, 15–17  $\text{mW}/\text{cm}^2$ ) for up to 1h. Pink line corresponds to photoinduced cytotoxicity.

Increasing DOX cellular uptake with increasing time of light activation of **1** was confirmed by FACS analysis (Figure S9) and to demonstrate the precision afforded by the described DOX delivery prodrug system, micelles of **1** were first incubated with cells then UV light applied over just half the well plate (Figure 4a). The result was clear spatial delineation of DOX cellular uptake *in vitro* (Figure 4b), highlighting not only efficient photolysis of **1** but also rapid cellular uptake of DOX once released.



**Figure 4.** Light templated doxorubicin delivery *in vitro*. Patterned light (365 nm, 15–17 mW/cm<sup>2</sup>) activation of **1** (300  $\mu$ M) and cellular uptake of DOX (**red**).

### 3.3 Conclusions

Here we demonstrate rapid and quantitative release of DOX from self-assembled micelles of **1** triggered by light. Prior to light activation, DOX-PEG conjugate based micelles are not cytotoxic, do not release DOX prematurely and share near identical physicochemical character to that of marketed and long-circulating liposome-DOX formulations (*e.g.* Doxil®). Towards tumor targeting of DOX *in vivo*, it is envisaged that micelles of **1**, administered systemically, will first passively accumulate within the tumor microenvironment via the EPR effect whereupon drug release could be triggered by light, on demand. Given the limited tissue penetration of single photon UV light, options to apply UV light to tumors residing deep within the body include the use of fiber-optic endoscopic techniques<sup>[15]</sup> or 2-photon light activation.<sup>[16]</sup> Alternatively, strategies rendering this system sensitive to longer wavelength, single photon, near-infrared (NIR) light can be considered.<sup>[17]</sup> Future studies will focus on the

application of these micelles *in vivo* and their potential use as an anti-cancer drug delivery system. In particular, care must be taken to maintain the concentration of **1** above the CMC following dilution in blood (approximately 5 L for an adult human).<sup>[18]</sup> For the system described, this equates to an injected dose of > 130 mg/5L of **1**—approximately 30 mg DOX. This figure is below the FDA recommended dosage for DOX·HCl (40–60 mg/m<sup>2</sup> administered every 21–28 days) currently used in the treatment of a wide range of human cancers.

### 3.4 Experimental

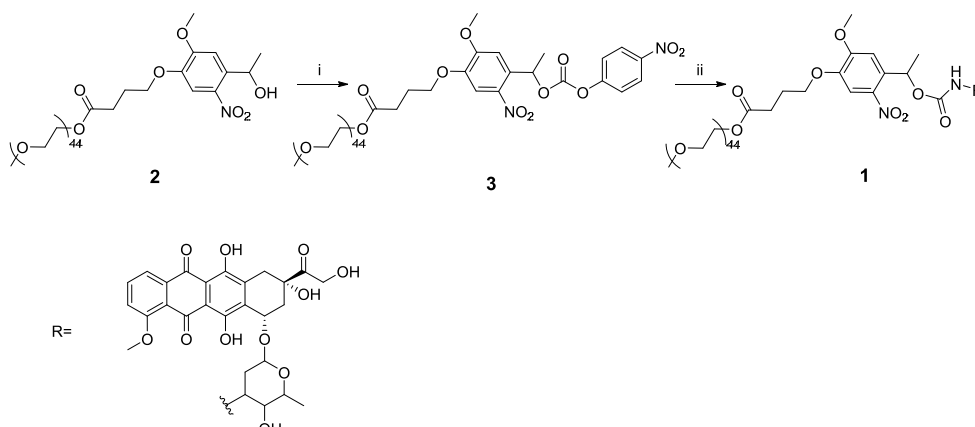
#### 3.4.1 Materials and Instruments

Doxorubicin hydrochloride (DOX·HCl) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA) and used without further purification. All other chemical reagents were purchased from Sigma-Aldrich (Zwijndrecht, Netherlands) and used without further purification. All solvents were purchased from Biosolve Ltd (Valkenswaard, Netherlands). Phosphate buffered saline (PBS): 5 mM  $\text{KH}_2\text{PO}_4$ , 15 mM  $\text{K}_2\text{HPO}_4$ , 150 mM NaCl, pH 7.4. Silica gel column chromatography was performed using silica gel grade 40–63  $\mu\text{m}$  (Merck & co., Amsterdam, Netherlands). TLC analysis was performed using aluminum-backed silica gel TLC plates (60F254, Merck, Amsterdam, Netherlands), visualization by UV absorption at 254 nm and/or staining with  $\text{KMnO}_4$  solution. NMR (nuclear magnetic resonance) spectra were measured on a AV-400MHz spectrometer (Bruker Nederland BV, Leiderdorp, MA, USA). Chemical shifts are recorded in ppm. Tetramethylsilane (TMS) is used as an internal standard. Coupling constants are given in Hz. LCMS analysis was performed on a Nanoacquity UPLC system-Synapt G2Si mass spectrometer (Waters Corporation, Milford, MA, USA) operating MassLynx software. Separation (Acquity UPLC M-Class 300  $\mu\text{m}$   $\times$  50 mm column, packed with BEH C4 material of 1.7  $\mu\text{m}$  diameter and 300Å pore size particles, flow rate: 2  $\mu\text{L}/\text{min}$ ; Waters Corporation, Milford, MA, USA) was carried out over a linear gradient of 10–90% **B** over 20 min. Buffers: **A**— $\text{H}_2\text{O}$  (0.1% Formic Acid); **B**—Acetonitrile (0.1% Formic Acid). Electro-spray ionization (ESI) via Nano-spray source with ESI emitters (New Objective Inc., Woburn, MA, USA) fused silica tubing 360  $\mu\text{m}$  OD  $\times$  25  $\mu\text{m}$  ID tapered to  $5 \pm 0.5$   $\mu\text{m}$  (5 nL/cm void volume). MS (mass spectrometry) settings (positive resolution mode): source temperature of 80 °C, capillary voltage 4.5 kV, nano flow gas of 0.25 Bar, purge gas 250 L/h, trap gas flow 2.0 mL/min, cone gas 100 L/h, sampling cone 25 V, source offset 25, trap CE 32 V, scan time 3.0 sec, mass range 400–2400  $m/z$ . Lock mass acquiring was done with a mixture of Leu-Enkephalin (556.2771) and [Glu1]-fibrinopeptide B (785.84265), lockspray voltage 3.5 kV, [Glu1]-fibrinopeptide B fragmentation was used as calibrant. MaxEnt 1 was used for mass deconvolution of the envelopes (Cambridge, UK). HPLC (high-performance liquid chromatography) analysis was performed using a Shimadzu

HPLC setup equipped with two LC-8A series pumps (Shimadzu Europa GmbH, 's-Hertogenbosch, Netherlands). Separation: Prep (Kinetex EVO, C18 column, 5u, 150 × 21.2 mm, flow rate: 15 mL/min; Phenomenex B.V., Utrecht, Netherlands), analytical (Vision HT, C18 column, 5 u, 150 × 4.6 mm, flow rate: 1 mL/min; Phenomenex B.V., Utrecht, Netherlands), in all instances, was carried out over a linear gradient of 10–95% **B** over 25 min with an initial 5 min hold at 10% **B**. HPLC buffers: **A**—H<sub>2</sub>O (0.1% TFA); **B**—Acetonitrile (0.1% TFA). UV detection at 254 nm.

For experiments not involving cells, UV light irradiation was performed using a hand-held BLAK-RAY B-100AP high intensity UV lamp (365 nm, 100 W; Fisher Scientific, Hampton, NH, USA) encased in a cardboard box. Samples were irradiated in quartz cuvettes at a fixed distance of 10 cm from the UV source. For all cell experiments, UV light irradiation was performed using a high-power LED (365 nm, 15–17 mW/cm<sup>2</sup>, Roithner Laser Technik GmbH, Vienna, Austria) mounted at a fixed distance of 1 cm above the cells.

### 3.4.2 Synthesis of 1



**Scheme S1.** Synthetic scheme to **1**. (i) 4-nitrophenylchloroformate, Et<sub>3</sub>N, CH<sub>2</sub>Cl<sub>2</sub>. (ii) Et<sub>3</sub>N, DMF.

**MethoxyPEG<sub>2000</sub> 4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanoate (2)** was synthesized as the method in chapter 2.

**MethoxyPEG<sub>2000</sub> 4-(2-methoxy-5-nitro-4-(1-(((4-nitrophenoxy)carbonyl)oxy)ethyl)phenoxy)butanoate, 3**

To a stirred solution of **2** (500 mg, 0.22 mmol) and 4-nitrophenyl chloroformate (265

mg, 1.31 mmol, 6 eq.) in CH<sub>2</sub>Cl<sub>2</sub> (20 mL) was added Et<sub>3</sub>N (305  $\mu$ L, 2.19 mmol, 10 eq.). The reaction mixture was stirred at room temperature in the dark overnight. Following solvent removal *in vacuo*, purification by column chromatography (*Gradient*: CH<sub>2</sub>Cl<sub>2</sub> to 15% MeOH in CH<sub>2</sub>Cl<sub>2</sub>) afforded **3** (278 mg, 0.11 mmol, 52%) as a yellow powder. **R<sub>f</sub>**: 0.30 (CH<sub>2</sub>Cl<sub>2</sub>:MeOH; 12:1). <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 400 MHz): 8.26 (d, *J* = 8 Hz, ArH-*o*-NO<sub>2</sub>, 2H); 7.61 (s, ArH-*o*-NO<sub>2</sub>, 1H); 7.35 (d, *J* = 8 Hz, ArH-*m*-NO<sub>2</sub>, 2H); 7.11 (s, ArH-*m*-NO<sub>2</sub>, 1H); 6.52 (q, *J* = 8 Hz, CH(CH<sub>3</sub>)OCOO, 1H); 4.26 (m, COOCH<sub>2</sub>CH<sub>2</sub>O, 2H); 4.14 (t, *J* = 8 Hz, OOCCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>O, 2H); 4.00 (s, CH<sub>3</sub>O, 3H); 3.45-3.95 (m, OCH<sub>2</sub>CH<sub>2</sub>, 196H); 3.32 (s, CH<sub>3</sub>OCH<sub>2</sub>CH<sub>2</sub>O, 3H); 2.59 (m, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>O, 2H); 2.19 (m, COOCH<sub>2</sub>CH<sub>2</sub>O, 2H); 1.78 (d, *J* = 8 Hz, CH(CH<sub>3</sub>)OCOO, 3H).

**MethoxyPEG<sub>2000</sub>4-(4-(1-(((3-hydroxy-2-methyl-6-(((1S,3S)-3,5,12-trihydroxy-3-(2-hydroxyacetyl)-10-methoxy-6,11-dioxo-1,2,3,4,6,11hexahydrotetracen-1-yl)oxy)tetrahydro-2H-pyran-4yl)carbamoyl)oxy)ethyl)-2-methoxy-5-nitrophenoxy)butanoate, **1****

To a stirred solution of **3** (86 mg, 0.034 mmol) and doxorubicin.HCl (20 mg, 0.037  $\mu$ mol) in DMF (500  $\mu$ L) was added Et<sub>3</sub>N (47.2  $\mu$ L, 0.34 mmol, 10 eq.). The reaction mixture was stirred at RT in the dark overnight. CH<sub>2</sub>Cl<sub>2</sub> (20 mL) was then added to the reaction mixture and the solution washed with brine (15 mL). The organic fraction was dried (Na<sub>2</sub>SO<sub>4</sub>) and solvent removed *in vacuo*. Column chromatography (*Gradient*: CH<sub>2</sub>Cl<sub>2</sub> to 2% MeOH in CH<sub>2</sub>Cl<sub>2</sub> to 10% MeOH in CH<sub>2</sub>Cl<sub>2</sub>) yielded **1** (58.1 mg, 61%) as a red powder. **R<sub>f</sub>**: 0.20 (CH<sub>2</sub>Cl<sub>2</sub>:MeOH; 12:1). <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 400 MHz): Partial peak assignment annotated in S3. <sup>1</sup>H-NMR of DOX with partial peak assignment included in S2. **MS** – despite numerous attempts to characterize this compound (MALDI, ESI), MS data was inconclusive – most likely due to compound instability and/or poor ionization of this compound during mass spec analysis. Following UV irradiation however, the MS of the photolysis products could be clearly detected (Figure S5 and S6). These products – nitroso-PEG and DOX – can only arise from the photolysis of **1**.

### 3.4.3 Preparation and Characterization of Light-Activated DOX-PEG Prodrug Micelles

Micelles of **1** were prepared via thin film hydration followed by sonication. Bath sonication (Branson 2510 Ultrasonic Cleaner, Branson Ultrasonics, Danbury, CT, USA) was carried out at 50 °C for 5 min. Particle size distributions were determined using a

Malvern Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, UK) equipped with a peltier controlled thermostatic holder, a fixed wavelength at 633 nm and scattering angle of 173°. DLS measurements were carried out at room temperature. For TEM observation, a drop of **1** (300 µM) was placed onto a nitrocellulose membrane covered TEM copper grid and dabbed dry through the underside of the grid with a tissue. This was then washed three times with ddH<sub>2</sub>O. A drop of uranyl acetate (2% w/v) in H<sub>2</sub>O was then added and the sample left to dry in the dark. Transmission electron microscopy (TEM JEOL 1010; *JEOL Ltd.*, Tokyo, Japan; Nieuw-Vennep, Netherlands) was run at an accelerating voltage of 60 kV.

#### **3.4.4 *In vitro* Drug Release**

To monitor the release profile of DOX following light irradiation, 1 mL of **1** (300 µM, > CMC) in PBS were placed in dialysis tubing (MWCO: 3.5 KDa) and dialyzed against 10 mL of dialysis buffers (PBS + 0.5% (w/w) Tween 80). At various time intervals, 3.0 mL of dialysis buffer was removed and replaced with fresh buffer. The amount of free DOX was quantified by UV–Vis absorbance measurements at 480 nm. To monitor light activated release of DOX, a sample of **1** was removed from the dialysis tubing at 9 h and irradiated for 30 min. This solution was returned to the dialysis tubing and the experiment continued. As a positive control, free DOX (300 µM) in PBS was subjected to the identical experimental conditions.

#### **3.4.5 WST Cell Proliferation Assay**

HeLa cells were seeded in 96-well plates at a density of 10000 cells per well and incubated overnight. Cells were washed once with PBS, then micelles of **1** (100 µL, varying concentrations in 1:1 PBS:DMEM+FCS), free DOX solutions (100 µL varying concentrations in 1:1 PBS:DMEM+FCS) or DMEM+FCS alone (100 µL) were added and the cells incubated for 12 h. Cells were then washed three times (DMEM+FCS), fresh DMEM+FCS added and incubated for a further 24 h. Cell media was removed and 200 µL Cell Proliferation Reagent; WST-1 (Sigma Aldrich, Zwijndrecht, Netherlands) added to each well. Cells were incubated (37 °C) for a further 3 h, according to the supplier guidelines. To determine cell viability, absorbance at 450 nm was measured. All experiments were carried out in quadruplicate.

#### **3.4.6 FACS Analysis**

HeLa cells were incubated with **1** (300  $\mu$ M in PBS, > CMC) for 30 min then irradiated (365 nm, 15–17 mW/cm<sup>2</sup>) for 15 min. Following irradiation, the solution was carefully removed, cells washed with PBS, trypsinized and immediately analyzed by flow cytometry. Counting and characterization was performed by measuring 10,000 events in triplicate and concatenation of this data. For manual gating, the outermost ring of the dot plot was selected. Quadrants were manually selected to illustrate fluorescence plots. No compensation was required.

#### **3.4.7 Light Templated DOX Delivery to Cells**

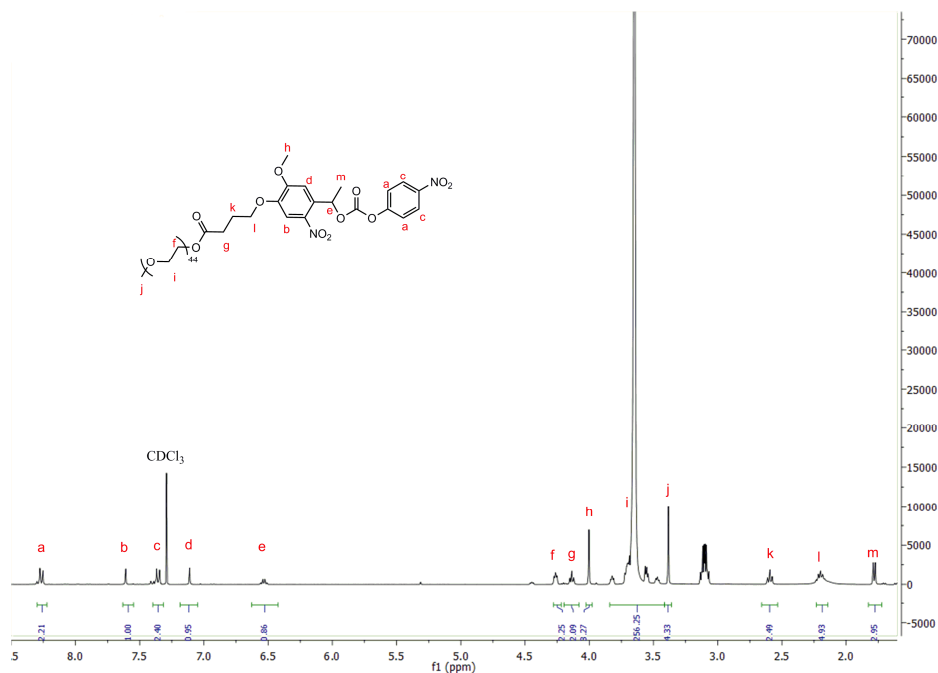
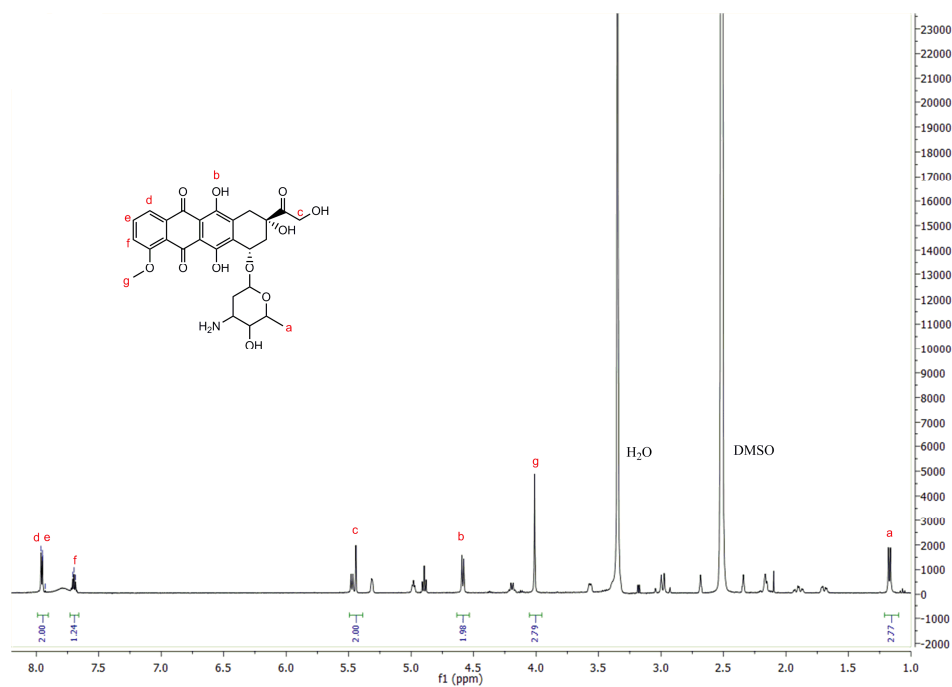
HeLa cells were seeded in 24-well plates (6  $\times$  10<sup>4</sup> cells per well) and incubated overnight. Cells were washed once with PBS, then micelles of **1** (300  $\mu$ M in PBS, > CMC) added and incubated for 30 min. Next, half of the well was covered with aluminum foil followed by UV irradiation (365 nm, 15–17 mW/cm<sup>2</sup>) from above for 15 min. Following irradiation, the solution was carefully removed, cells washed (3  $\times$  DMEM+FCS) and immediately analyzed under the fluorescence microscope.

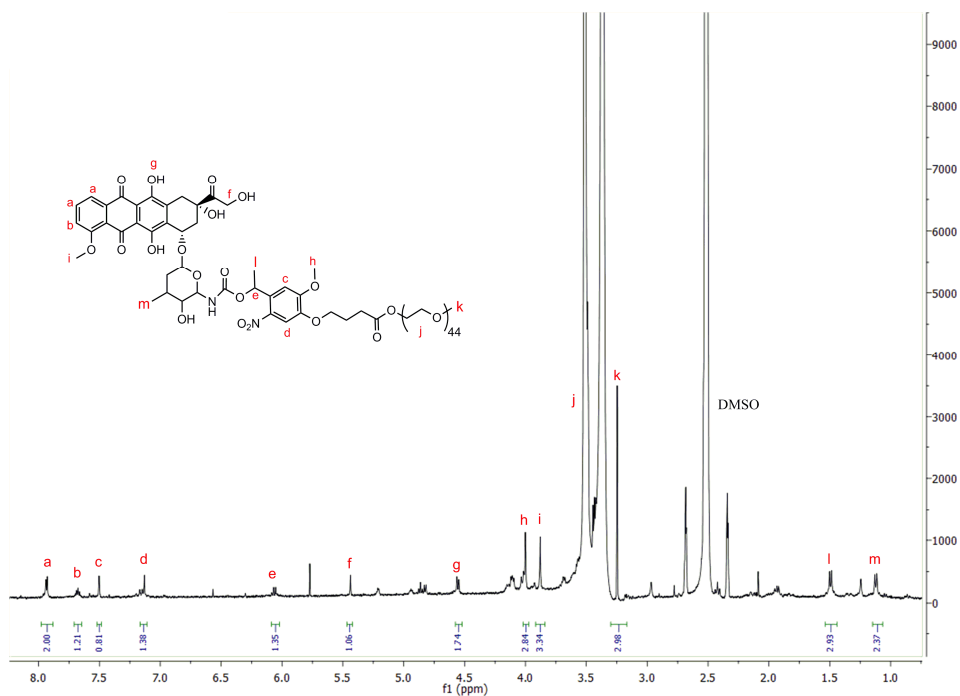
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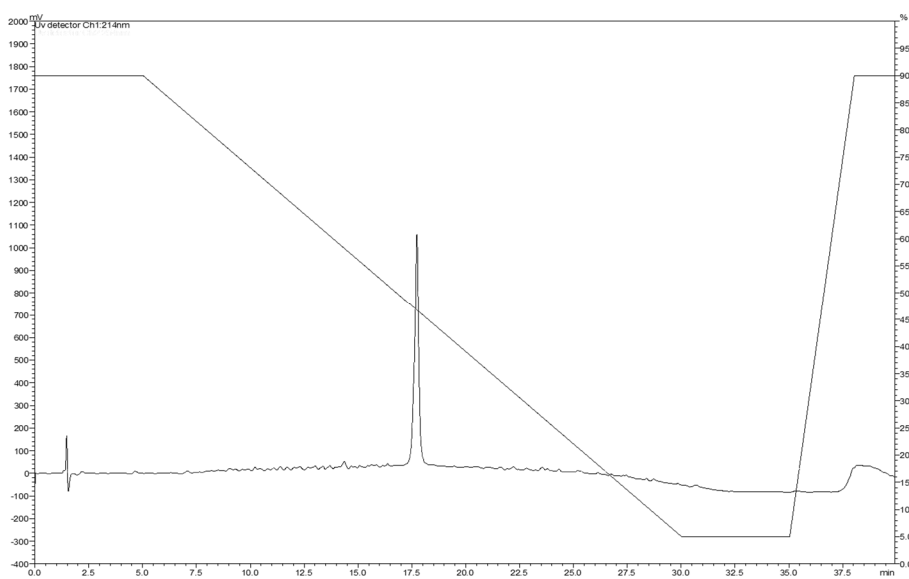
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## 3.6 Appendix

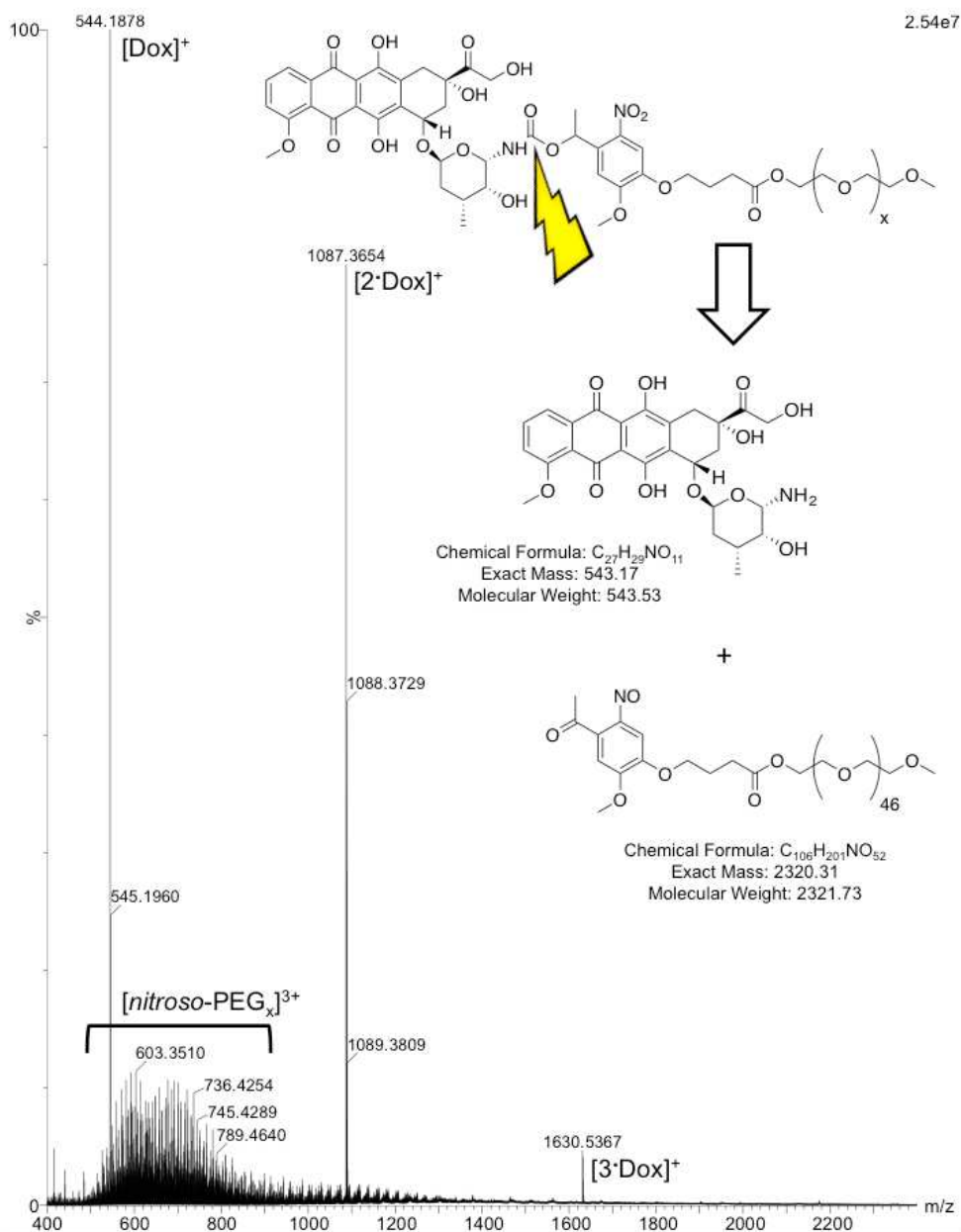
Figure S1.  $^1\text{H}$ -NMR of **3**.Figure S2.  $^1\text{H}$ -NMR of Doxorubicin.



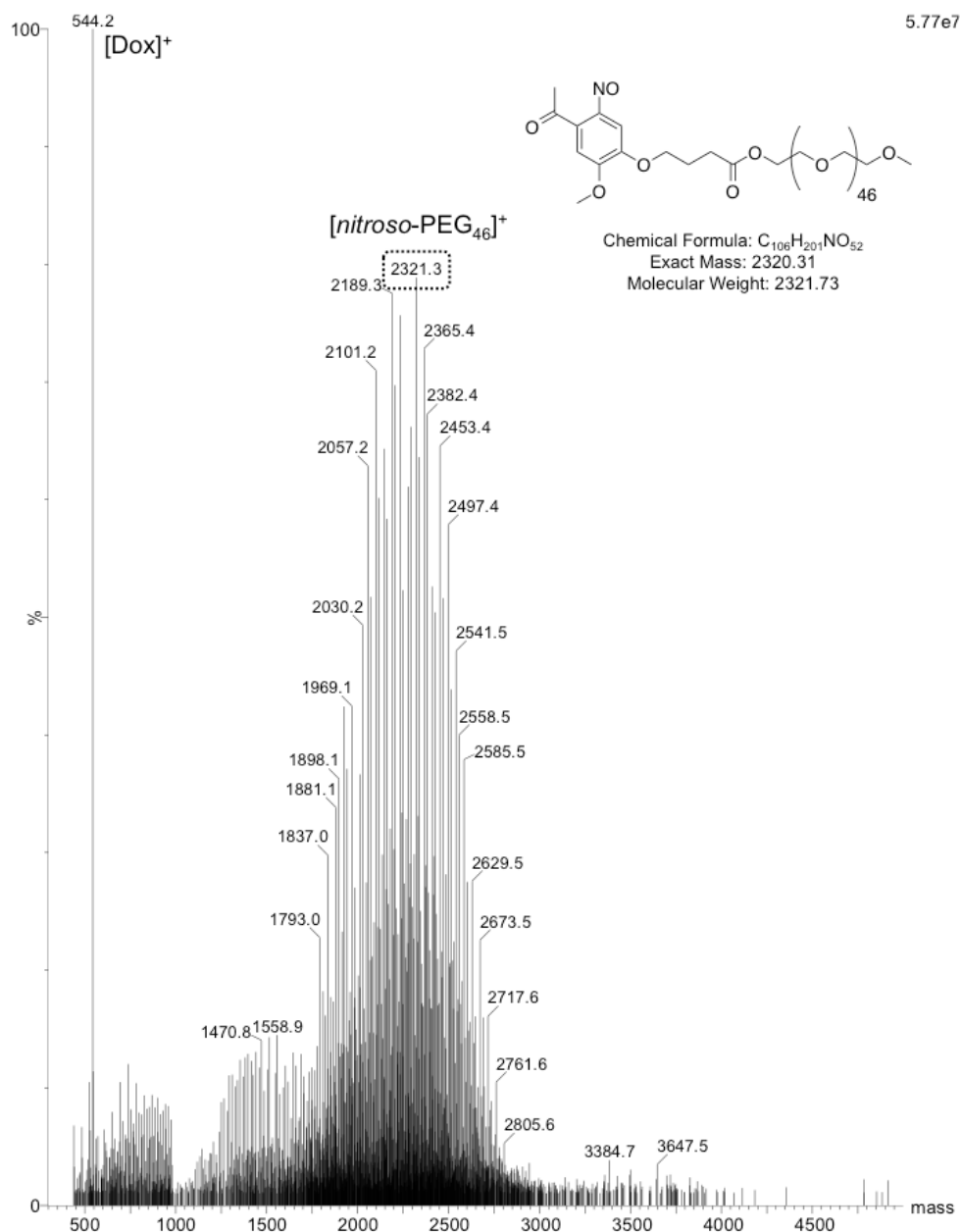
**Figure S3.**  $^1\text{H}$ -NMR of **1**.



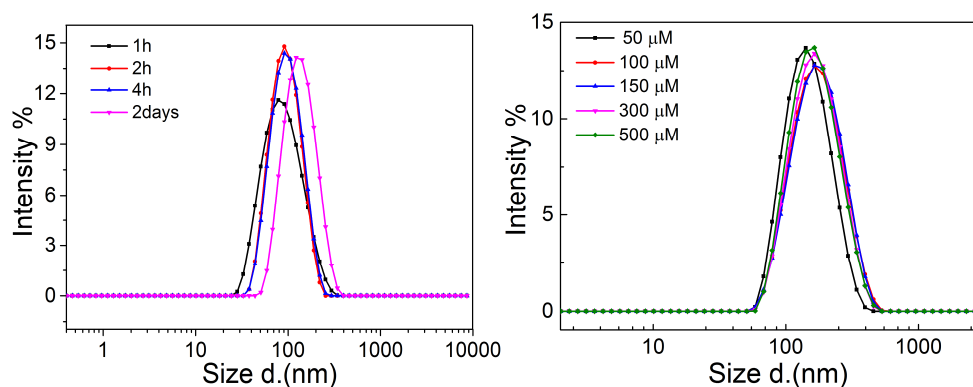
**Figure S4.** HPLC trace of **1**. Retention time – 17.8 min. UV detection – 214 nm.



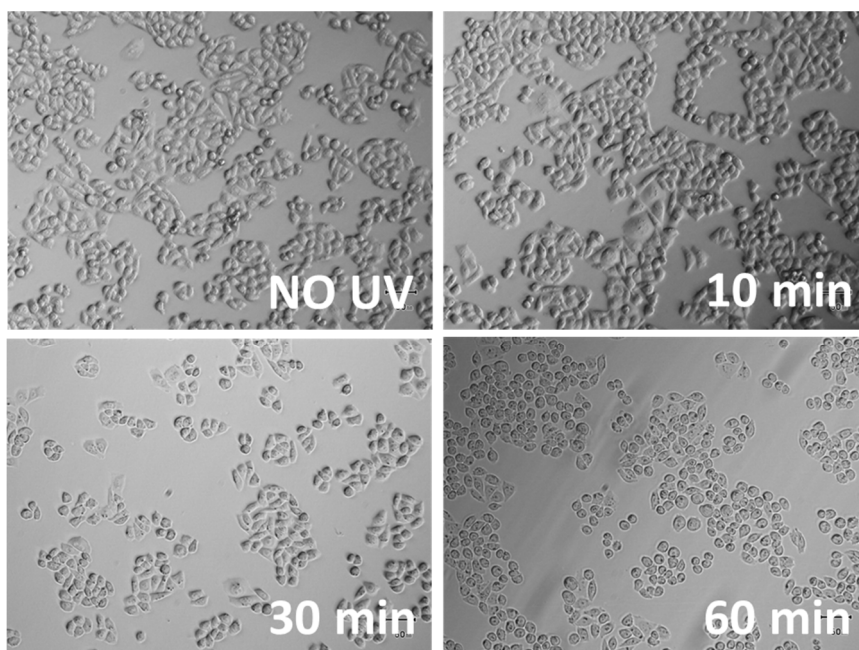
**Figure S5.** ESI-MS spectra (raw data) following photolysis of **1** and showing the expected photoproducts – DOX and nitroso-benzyl-PEG2000 – as the only significant species present. The presence of DOX clusters –  $[2 \cdot DOX]^+$  and  $[3 \cdot DOX]^+$  - in the raw spectra arise from ‘soft’ electrospray ionization techniques.



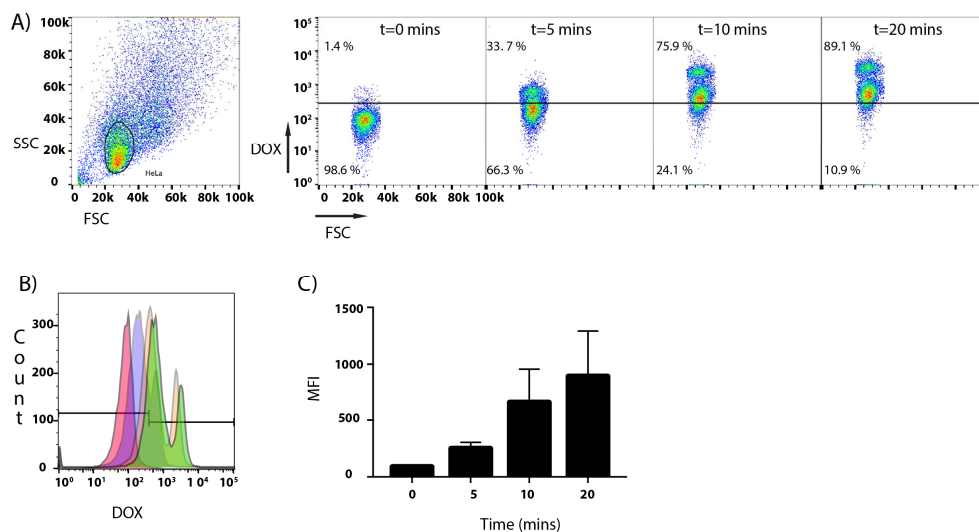
**Figure S6.** Deconvoluted (software: MaxEnt1) mass spectra of nitroso-PEG envelope signals.



**Figure S7.** (left) Time course DLS size distributions of **1** (300 μM in PBS) diluted (1:1) in DMEM+FCS. (right) DLS size distributions of **1** (varying concentrations) in PBS.



**Figure S8.** Cells (bright field) irradiated for varying times (UV-A, 365 nm, 15-17 mWcm<sup>-2</sup>) and imaged immediately. As UV-A irradiation times increase cells become smaller (shrinkage) and more rounded, hallmarks of the onset of UV-A induced apoptosis.



**Figure S9.** FACS analysis showing increased uptake of DOX (released from a solution of **1** (300  $\mu$ M in PBS)) by HeLa cells with increasing irradiation times. A) Dot plots of HeLa cells after  $t=0$ , 5, 10 and 20 min of irradiation; cell population was gated based on FSC-A vs SSC-A (cell doublets were gated out using FSC-A vs FSC-H). B) Histograms of HeLa cells after  $t=0$  min (pink)  $t=5$  min (blue),  $t=10$  min (orange) and  $t=20$  min (green) irradiation. C) Mean Fluorescence Intensity (MFI) of HeLa cells after different irradiation times. Error bars  $\pm$  SD.

