Published in final edited form as:

ACS Appl Mater Interfaces. 2019 January 23; 11(3): 2814–2820. doi:10.1021/acsami.8b18099.

Polymer Amphiphiles for Photoregulated Anticancer Drug Delivery

Valentina Brega^a, Federica Scaletti^b, Xianzhi Zhang^b, Li-Sheng Wang^b, Prudence Li^c, Qiaobing Xu^c, Vincent M. Rotello^b, and Samuel W. Thomas III^{*,a}

^aDepartment of Chemistry, Tufts University, 62 Talbot Avenue, Medford MA 02155, United States.

^bDepartment of Chemistry, University of Massachusetts Amherst, 710 Nt. Pleasant Street, Amherst MA 01003, United States.

^cDepartment of Biomedical Engineering, Tufts University, 4 Colby Street, Medford MA 02155, United States.

Abstract

We report the synthesis of amphiphilic polymers featuring lipophilic stearyl chains and hydrophilic poly(ethylene glycol) (PEG) polymers that are connected through singlet oxygen-cleavable alkoxyanthracene linkers. These amphiphilic polymers assembled in water to form micelles with diameters of ~20 nm. Reaction of the alkoxyanthracene linkers with light and O₂ cleaved the ether C-O bonds, resulting in formation of the corresponding 9,10-anthraquinone derivatives and concomitant disruption of the micelles. These micelles were loaded with the chemotherapeutic agent doxorubicin, which was efficiently released upon photo-oxidation. The drug-loaded reactive micelles were effective at killing cancer cells *in vitro* upon irradiation at 365 nm, functioning through both doxorubicin release and photodynamic mechanisms.

Graphical Abstract

Keywords

Self-assembly; light-responsive materials; amphiphilic polymers; drug delivery; doxorubicin

*Corresponding Author: sam.thomas@tufts.edu (S.W.T.).

ASSOCIATED CONTENT

Supporting Information.

The following files are available free of charge.

¹H and ¹³C NMR spectra, MALDI-TOF mass spectra, UV-Vis measurements, Irradiation experiments, Control experiments, DOX loading and release experiments, Cell viability, Cellular uptake, *in vitro* irradiation experiments (PDF)

Introduction

Materials that degrade upon application of specific chemical or physical triggers have potential for on-demand delivery,¹ and other applications.^{2,3} A range of responsive linkers cleave upon application of a specific endogenous stimulus, such as disulfides for chemical reduction⁴ or acetals for hydrolytic degradation.⁵ Photodegradable materials provide an external stimulus for activation, harnessing the unique advantages of light, including spatiotemporal precision and the ability to penetrate through barriers impermeable to traditional chemical reagents. Photocleavable groups are common components of photodegradable macromolecular architectures,^{6,7} such as UV-disruptable block copolymer micelles that release Nile Red upon irradiation.⁸ Furthermore, a number of approaches are applicable to drug delivery systems that respond to visible or NIR light,⁹ such as two-photon absorption,^{10,11} photothermal heating^{12,13,14} and upconverting nanoparticles.^{15,16,17,18,19}

As an alternative, the reactive oxygen species (ROS) singlet oxygen ($^1\text{O}_2$) is both readily generated photochemically *and* can cleave specific chemical bonds. $^1\text{O}_2$ engages in [2+2] cycloadditions and ene reactions, and can also act as a dienophile, oxidizing anthracenes and other polycyclic aromatic hydrocarbons to endoperoxides through [4+2] cycloaddition reactions.^{20,21,22} The cytotoxic nature of $^1\text{O}_2$ makes it important in photodynamic therapy, a treatment that uses a photosensitizer to produce ROS upon irradiation to destroy cancer cells.²³ The ubiquity and ease of generation of $^1\text{O}_2$ has led to the concept of $^1\text{O}_2$ cleaving chemical bonds selectively. Beyond the initially reported electron-rich alkenes, other moieties have emerged that undergo bond cleavage upon reaction with $^1\text{O}_2$, such as aminoacrylates,^{24,25,26,27,28} alkoxyanthracenes,^{29,30,31,32,33,34,35} and thioketals.^{36,37} These classes of $^1\text{O}_2$ -cleavable groups have been integrated into a range of degradable materials, including block copolymer micelles,^{38,39} nanoparticles,⁴⁰ nanorods,⁴¹ and vesicles.⁴² Two principal methods exist that harness $^1\text{O}_2$ -responsive linkers for applications in drug delivery: i) materials in which a prodrug is covalently linked through a $^1\text{O}_2$ -cleavable linker,⁴³ and ii) materials in which the drug is non-covalently trapped in a $^1\text{O}_2$ -degradable carrier.³⁹

Cleavage of linkages between hydrophobic and hydrophilic segments of amphiphiles provides an effective strategy to disrupt micelles.³⁹ Herein we report $^1\text{O}_2$ -cleavable amphiphiles featuring alkoxyanthracene derivatives that bridge lipophilic and hydrophilic segments of polymers that assemble into photooxidatively degradable micelles. We prepared two amphiphilic polymers (**9,10-C₁₈PEG** and **3,9-C₁₈PEG**), each consisting of a lipophilic stearyl chain and a hydrophilic polyethylene glycol (PEG) polymer connected through a $^1\text{O}_2$ -cleavable linker (Figure 1). We demonstrate that photochemical oxidation of these polymers results in micellar disruption due to the oxidation and subsequent bond cleavage of the linker between the hydrophobic and hydrophilic segments of the polymers, yielding responsive assemblies. These polymers provided a photochemically-activated therapeutic platform that combines chemotherapeutic payload release with photodynamic therapy.

Experimental Section

General Information.

^1H and ^{13}C NMR spectra were acquired with a Bruker AVANCEIII 500 MHz NMR, in deuterated solvent at room temperature. Chemical shifts are given in parts per million (ppm). All spectra were processed with Topspin 2.1 (Bruker Biospin) and further visualized with MestreNova, version 12 (Mestrelab Research, Santiago de Compostela, Spain).

All reactions were monitored using silica gel 60 F254 analytical TLC plates with UV detection ($\lambda = 254$ nm and 365 nm). Silica gel (60 Å, 40–63 μm) was used as the stationary phase for column chromatography. The spectrophotometric measurements of compounds were carried out in solvents of spectrophotometric quality. UV-vis absorption spectra were recorded using a Varian Cary-100 spectrophotometer in double beam mode. Irradiation experiments were performed with a 200 W Hg/Xe lamp (Newport-Oriel) equipped with a water filter, manual shutter, focusing lens, and the appropriate wavelength selecting filters. For irradiation at 365 nm, a combination of a 295 nm long-pass filter and a 365 nm band-pass filter was used, giving an average power density of 38 mW/cm². For longer wavelength irradiations at $\lambda > 630$ nm, a 630 nm long-pass filter was used, giving an average power density of 50 mW/cm². For irradiation at $\lambda > 495$ nm, two 495 nm long-pass filters were used, giving an average power density of 71 mW/cm².

Dynamic light scattering (DLS) measurements were performed on a Malvern Zetasizer Nano-ZS (Malvern Instrument Ltd., U.K.) equipped with a He–Ne laser operating at 633 nm at 25 °C. Samples were prepared in pure water and filtered through 0.2 μm PTFE syringe filters before measurements. Mass spectrometry of polymers was performed using a Bruker Microflex MALDI-TOF mass spectrometer.

High-resolution mass spectral (HRMS) analyses of new compounds were performed by the Massachusetts Institute of Technology mass spectrometry facility using electrospray ionization in positive mode.

Transmission Electron Microscopy (TEM).

Freshly prepared sample solution was drop-cast onto a TEM grid (carbon film, 400 mesh copper, Electron Microscopy Sciences), and the sample was allowed to dry at room temperature overnight. Negative staining was employed to enhance the imaging contrast.⁴⁴ 2% uranyl acetate solution was dropped on the sample for 30 seconds, following by drying using a filter paper. The structural of samples were inspected using a JEOL 2000FX TEM with an accelerating voltage of 200 kV.

Chemicals.

All starting materials and solvents were purchased from Sigma-Aldrich, TCI Chemicals, or Fisher Scientific and, unless otherwise specified, were used without further purification. Deuterated solvents were purchased from Cambridge Isotope Laboratories. mPEG-Amine and mPEG-OC18 (MW 2000 Da) were purchased from Creative PEGWorks. Doxorubicin, hydrochloride salt was purchased from LC Laboratories. 9,10-dimethoxyanthracene,⁴⁵ 9-

methoxyanthracene,⁴⁶ cis-1,2-diphenyldithioethene⁴⁷ were synthesized according to literature procedures.

Results and Discussion

A number of peroxides that result from cycloaddition of $^1\text{O}_2$ are reported to cleave bonds in the original reactant; such moieties have the potential to be $^1\text{O}_2$ -cleavable groups in polymer materials. To inform designs of our $^1\text{O}_2$ -cleavable amphiphiles, we first compared the reactivities of a range of reported $^1\text{O}_2$ -cleavable groups by monitoring their disappearance in CH_2Cl_2 by UV-vis spectrophotometry upon irradiation of the sensitizer methylene blue (Figure 2). We analyzed the kinetics of disappearance using a pseudo first-order kinetic model for the disappearance of 9,10-dimethoxyanthracene, 9-methoxyanthracene, cis-1,2-diphenyldithioethene, and ethyl β -dimethylamino acrylate, all relative to a standard for chemical reactions with $^1\text{O}_2$, 9,10-diphenylanthracene (DPA). The Supporting Information contains the spectroscopic data for each kinetic trace (Figure S11). While the dithioethene and aminoacrylate each reacted with half the rate of DPA, the electron rich methoxyanthracenes reacted faster than DPA, with 9,10-dimethoxyanthracene reacting four times faster than 9-methoxyanthracene. These observations agree with the known trend that more electron rich, readily oxidized acenes react faster with $^1\text{O}_2$ than less electron-rich acenes.

To support the hypothesis that $^1\text{O}_2$ is a key reactive oxygen species in these reactions of alkoxyacenes, we analyzed the products of photochemical oxidation of 9,10-dimethoxyanthracene under several conditions (See Figures S38–S42). Selective irradiation of methylene blue with $\lambda > 600$ nm in the presence of 9,10-dimethoxyanthracene in acetone- d_6 yielded the corresponding 9,10-endoperoxide, as would be expected upon cycloaddition with $^1\text{O}_2$ with no discernable byproducts by NMR spectroscopy. Especially key to making this assignment was the characteristic ^{13}C NMR signal for the bridgehead protons at 102.9 ppm. Similarly, direct irradiation of 9,10-dimethoxyanthracene at 365 nm yielded identical ^1H NMR and ^{13}C NMR spectra as selective irradiation of methylene blue (Figure S38–S42). We therefore conclude that irradiation of 9,10-dimethoxyanthracene produces $^1\text{O}_2$, which then undergoes [4+2] cycloaddition to yield the 9,10-endoperoxide.

We chose the two most reactive alkoxyanthracene derivatives as linking moieties in cleavable amphiphiles that contain PEG. In addition to being hydrophilic, PEG has the advantages of biocompatibility and resistance to fouling,^{48,49,50} as well the commercial availability of a variety of reactive derivatives. Scheme 1 summarizes our successful approach for synthesizing the target amphiphiles. The preparation of the 9,10-dialkoxyanthracene-linked amphiphile began with reductive alkylation of 9,10-anthraquinone. A mixture of *tert*-butylbromoacetate and 1-bromooctadecane gave the desired unsymmetric product **1**, followed deprotection with trifluoroacetic acid (TFA) to yield carboxylic acid **2**.⁵¹ Amidation of **2** with amine-functionalized PEG (2 kDa) under standard conditions led to the cleavable amphiphile **9,10-C₁₈PEG**. Following a similar strategy, reduction of 2-octadecyloxyanthraquinone⁵² gave the 3-alkoxy-9-anthrone regioisomer **3** selectively. Alkylation of **3** with *tert*-butylbromoacetate yielded the desired O-

alkylated compound **4**. After deprotection with TFA, EDC-promoted amidation with methoxy PEG amine (2 kDa) again yielded target amphiphilic polymer **3,9-C₁₈PEG**.

Amphiphiles **9,10-C₁₈PEG** and **3,9-C₁₈PEG** appeared soluble in water by visual inspection, forming optically clear, foamy solutions upon gentle shaking and filtration through 0.2 μ m PTFE filters. Dynamic light scattering (DLS) of these samples revealed the presence of particles with average hydrodynamic diameters of $\sim$ 20 nm for both polymers, which was corroborated by transmission electron microscopy (TEM, Figure 3 and Figure S24–S28). While **9,10-C₁₈PEG** forms only spherical micelles (Figure S26), **3,9-C₁₈PEG** forms mixtures of spherical and rod-like micelles (Figure S27 and S28), with a larger proportion of rod-like micelles at higher amphiphile concentrations. Previous literature indicates that spherical micelles aggregating at increasing concentration of non-ionic PEG-based surfactants causes this sphere-to-rod transition.⁵³ The sizes of these micelles were generally smaller than micelles prepared from block copolymers,^{38,39} and comparable to previously reported micelles comprising an *n*-C₁₈H₃₇ hydrophobic segment and PEG hydrophilic segment,⁵⁴ indicating that the anthracene linkers do not perturb micellization. Changes in the absorbance of eosin Y as a function of amphiphile concentration enabled estimation of the critical micelle concentrations (CMCs) of these polymers,⁵⁵ which we determined to be 64 μ M (0.16 mg/mL) for **9,10-C₁₈PEG** and 74 μ M (0.18 mg/mL) for **3,9-C₁₈PEG** (Figure 4). These values are largely indistinguishable to the CMC of a commercially available analog (**C₁₈PEG**) that does not include any anthracenes (89 μ M, Figure S13).

Upon irradiation at 365 nm, the decomposition of the linkers triggered the disruption of the micelles. We monitored photochemical conversion of these samples by both UV/vis spectrophotometry and DLS in deionized water. The characteristic absorbance bands of the anthracene chromophores decreased upon irradiation, with the anthracene in **9,10-C₁₈PEG** reacting $\sim$ 4-fold faster than that of **3,9-C₁₈PEG** (Figure S14 and S18), consistent with our earlier kinetics experiments in organic solvent. In addition, the optically transparent samples developed visible precipitate after photo-oxidation, which we ascribe to aggregates of hydrophobic photoproducts that contain anthraquinones, and in the case of **9,10-C₁₈PEG**, stearyl alcohol. The large and polydisperse nature of these aggregates made analysis of these “as-irradiated” samples by DLS impossible. After filtration, however, analysis of these samples by DLS showed partial photo-induced transformation of these small micelles into larger particles of average diameter between 100–200 nm, with the $\sim$ 20 nm-diameter micelles of **9,10-C₁₈PEG** disappearing completely after one hour, while micelles of **3,9-C₁₈PEG** were only partially consumed after two hours (Figure S15 and S19).

To establish the products of UV irradiation of these anthracene-containing amphiphiles when they are assembled into micelles, we irradiated micellar aqueous solutions of **9,10-C₁₈PEG** or **3,9-C₁₈PEG** with $\lambda = 365$ nm, followed by lyophilization and ¹H NMR analysis of the samples in CDCl₃, which dissolves all photoproducts. Inspection of the aromatic region of the spectra revealed 9,10-anthraquinone (Figure 5b) as the primary aromatic product of the irradiation of **9,10-C₁₈PEG** (Figure 5a), and 2-octadecyloxyanthraquinone (Figure 5d) as the primary aromatic product of the irradiation of **3,9-C₁₈PEG** (Figure 5c). These anthraquinone products are consistent with the proposed mechanism of photooxidation and cleavage of alkoxyacenes reported in the literature.^{29,56}

We therefore conclude that the bonds between the hydrophobic and hydrophilic segments of these amphiphiles cleave upon irradiation due to exposure to $^1\text{O}_2$, which leads to micelle disruption.

In an initial demonstration of phototriggered release, we loaded micelles comprising **9,10-C₁₈PEG**, **3,9-C₁₈PEG**, or **C₁₈PEG** with the guest doxorubicin (DOX), a hydrophobic chemotherapeutic drug.^{57,58,59} The release of DOX from the solubilizing environment of the micelle was monitored by the decrease of intensity of the DOX band in the UV-vis spectra of an aqueous solution of micelles loaded with solubilized DOX after irradiation ($\lambda = 365$ nm), and filtration with a 0.2 μm PTFE syringe filter to remove the de-solubilized DOX that aggregates upon micelle disruption. We ascribe the faster rate of DOX removal from **9,10-C₁₈PEG** micelles compared to **3,9-C₁₈PEG** and **C₁₈PEG** micelles (Figure 6 and Figure S33–S35) to faster decomposition of the 9,10-dialkoxy cleavable linkers.

A potential advantage of $^1\text{O}_2$ -responsive materials is the range of sensitizers that can produce $^1\text{O}_2$ upon irradiation with visible or near-infrared light. We used eosin Y as a photosensitizer, as Methylene Blue gave rise to larger aggregates according to DLS measurements, while Rose Bengal exhibited poor photostability. Irradiation of a solution containing both eosin Y and **9,10-C₁₈PEG** at $\lambda > 495$ nm yielded irreversible oxidation of the anthracene linker (Figure S16). DLS analysis after filtration did reveal partial transformation of the micelles into larger particles (Figure S17), but micelles were not completely consumed after one-hour irradiation as observed during exposure to UV light. Irradiation at $\lambda > 495$ nm in the absence of sensitizer yielded no anthracene oxidation (Figure S22). In addition, removal of O_2 from the micellar suspension slowed reaction of anthracene in **9,10-C₁₈PEG** dramatically, achieving only 20–25% conversion after 60 minutes (Figure S23); similar conversion in aerated water required less than 3 minutes (Figure S16).

To evaluate their therapeutic potential *in vitro*, we compared the extent to which these micelles reduced the viability of HeLa cells (Figure 7) when they were: i) either devoid of cargo or loaded with DOX, and ii) either kept in the dark or irradiated at 365 nm. Confocal microscopy of DOX-loaded micelles, using the intrinsic fluorescence of DOX as an imaging agent, confirmed cellular uptake of DOX loaded micelles into the cytosol of HeLa cells (Figure S37). Micelles comprising the control amphiphile **C₁₈PEG**, and therefore lacking any moiety that absorbs 365 nm showed minimal toxicity, regardless of whether they were irradiated, loaded with DOX, or both. In contrast, irradiation of micelles comprising either **9,10-C₁₈PEG** or **3,9-C₁₈PEG**, but not loaded with DOX, decreased cell viability to 44–56%, while identical samples kept in the dark did not show increased toxicity relative to the control amphiphile **C₁₈PEG**. We attribute this photoinduced cytotoxicity in the absence of DOX to $^1\text{O}_2$, and perhaps other ROS, formed upon direct irradiation of alkoxyacene chromophores. When loaded with DOX and irradiated with light, micelles comprising **9,10-C₁₈PEG** showed even greater toxicity by reducing cell viability to $27 \pm 3\%$, while the same sample in the absence of light showed minimal toxicity. We ascribe the increased phototoxicity of **9,10-C₁₈PEG** micelles compared to **3,9-C₁₈PEG** micelles to faster decomposition of the 9,10-dialkoxy cleavable linkers, yielding faster release of the DOX cargo, in accordance with the model release experiments (Figure 6). These results are

consistent with cooperative photoinduced cytotoxicity of drug-loaded **9,10-C₁₈PEG** micelles that combine the traditional photodynamic effect of ROS production with photo-triggered release of DOX. The estimated concentration of DOX used for *in vitro* delivery experiments (0.18 μ M) is lower than the IC₅₀ (50% of growth inhibition) that we measured for doxorubicin acting on HeLa cells (1.3 μ M), which is in accordance with the reported IC₅₀ (1–5 μ M),^{60,61} further suggesting the combination of ROS and released DOX as responsible for the increased cytotoxicity.

Conclusions

We have demonstrated the rational design of nanometric, photo-oxidatively degradable micelles containing alkoxyanthracene-based singlet oxygen cleavable groups, including a 9-alkoxy-10-hydroanthracene linker. The products of irradiation of these micelles in aqueous solution are consistent with reaction with singlet oxygen to form endoperoxides, followed by bond cleavage to yield the corresponding quinones. The reactivity of the anthracene moieties controls the kinetics of micelle degradation, the rate of cargo release, and the observed photoinduced cytotoxicity. From a materials design perspective, our results provide a flexible platform for the discovery of nanoscale delivery vehicles that exhibit enhanced photodynamic therapy—the combination of photodynamic therapy with photo-responsive materials that can release a trapped drug—while laying the groundwork for potential paths forward for harnessing visible or NIR light through either the integration of a separate sensitizer or modifications of alkoxyacenes to absorb longer wavelengths. More fundamentally, this work also highlights the value of continuously improving our understanding of chemical reactivity for the rational design of responsive nanomaterials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

Funding from the NIH (EB022207) supported the structure-property studies and model release experiments, while funding from the NSF (CHE-1609146, to ST) and the NIH (EB02264, to VR) supported the fluorescence microscopy, transmission electron microscopy, and *in vitro* experiments. We thank James Chambers (Light Microscopy Facility at UMass Amherst) for assistance with confocal laser scanning microscopy.

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Scheme 1.

Synthesis of the amphiphilic polymers **9,10-C₁₈PEG** and **3,9-C₁₈PEG**, and chemical structure of commercially available, unreactive amphiphile **C₁₈PEG**. In each case, the molecular weight of PEG was 2 kDa.

Figure 1.

a) Photooxidation and cleavage reaction of the amphiphilic polymer **9,10-C₁₈PEG**. *b)* Schematic depiction of micelle disruption upon anthracene photooxidation. *c)* Intracellular DOX release.

Figure 2. Pseudo-first-order kinetics of reaction of four reported $^1\text{O}_2$ -cleavable groups upon irradiation of methylene blue in CH_2Cl_2 ; $k_{\text{rel}} = 1$ for 9,10-diphenylanthracene.

Figure 3.

Left: Distribution of hydrodynamic diameters of **9,10-C₁₈PEG** and **3,9-C₁₈PEG** micelles in water (1.0 mg/mL), determined by dynamic light scattering. *Right:* Negative stain TEM images of **9,10-C₁₈PEG** and **3,9-C₁₈PEG** micelles (1.0 mg/mL and 0.5 mg/mL respectively).

Figure 4.

Sequences of spectra recorded over the course of titrating a 7.0 μM solution of eosin Y with a solution of 9,10-C₁₈PEG (top) or 3,9-C₁₈PEG (bottom): spectrum of eosin Y prior to the addition (thick black line); spectrum after the addition of polymer (thick blue line). The inset shows titration profiles at 542 nm and determination of CMCs.

Figure 5. Aromatic region of ^1H NMR spectra in CDCl_3 of a) $9,10\text{-C}_{18}\text{PEG}$ before and b) after one hour UV irradiation and c) $3,9\text{-C}_{18}\text{PEG}$ before and d) after 12 hours UV irradiation.

Figure 6.

Release of DOX from micelles in water upon exposure to light ($\lambda = 365$ nm) and in the dark at room temperature. The amount of DOX removed from the solubilizing environment of the micellar cores upon irradiation of 9,10-C₁₈PEG micelles is greater than the amount released from 3,9-C₁₈PEG and C₁₈PEG micelles. Error bars show standard errors of three replicates.

Figure 7.

Cell viability of HeLa cells upon exposure to micelles that are either: i) loaded with DOX (YES) or not loaded (No), and ii) irradiated at 365 nm in the presence of the cells (Light) or not irradiated (Dark). Error bars show standard deviations of four replicates. The irradiation time for all “light” experiments was 60 minutes.