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Nanometric Micelles with Photo-Triggered Cytotoxicity

Parambath Anilkumar, Edmond Gravel, Ioanna Theodorou, Karine Gombert, Benoit Thézé, Frédéric Ducongé, and Eric Doris**

The development of a photo-responsive micellar system capable of triggering cell death is reported. Precursors of the micelles are synthesized by connecting a lipophilic chain to a hydrophilic polyethylene glycol via a photolabile nitrobenzyl group. The resulting amphiphilic units are self-assembled in water forming 12 nm micelles that are readily internalized into cells. Upon photo-irradiation, micelles undergo cleavage and yield a cytotoxic nitrosobenzaldehyde derivative, which significantly inhibits the proliferation of MDA-MB-231 cells under standard in vitro conditions.

1. Introduction

The potential of nanotechnology products applied to medicine has led to the development of nanometric drug carriers[1] such as liposomes,[2] dendrimers,[3] polymers,[4] nanoparticles,[5] and nanometer-sized micelles.[6] The latter species have emerged as highly sought materials for drug delivery applications and have gathered considerable attention because of easy design, facile synthesis, and fine tuning of shape and aggregation properties.[7] However, micellar delivery systems can also have some drawbacks such as side accumulation in vital organs and burst release.[8] Unwanted accumulation can be minimized using polyethylene glycol (PEG) coated micelles that are considered as promising carriers because of their favorable pharmacokinetics.[9] Furthermore, therapeutic effectiveness can be improved by controlling the specific delivery of therapeutics to the site of action. This can be achieved using stimuli-responsive carriers that are sensitive to either temperature, pH, oxidation/reduction, or light.[10] Since light can be localized in time and space, and applied from outside of the system, introduction of photoresponsive groups in the micelle architecture appears to be an attractive strategy for triggered therapeutic effects.[11] Among the various photolabile groups, nitrobenzyl (NB) derivatives have gained considerable attention both as organic protecting groups[12] and as photo-cleavable linkers for hydrogels,[13] block copolymers,[14] and bioconjugates[15] for biomedical applications. In a preliminary study, Zhao and coworkers confirmed the light-induced disassembly of NB-based block copolymer micelles by monitoring the release of a payload encapsulated in the hydrophobic core.[14] Furthermore, Rotello and coworkers induced the in vitro release of an anticancer drug (5-fluorouracil) conjugated to gold nanoparticles.[15]

The mechanism behind the photo-induced cleavage is based on a cascade of reactions resulting in the formation of aromatic nitrosobenzaldehyde together with the release of the free carboxylic acid partner.[16] Previous reports demonstrated that aromatic nitroso compounds can be toxic to cells. Indeed, *C*-nitroso compounds readily form adducts with many cysteine-containing proteins inhibiting enzymatic or cellular activity.[17] For example, Kun and coworkers observed that simple aromatic nitroso compounds (e.g., 6-nitroso-1,2-benzopyrone and 3-nitrosobenzamide) can significantly inhibit poly(ADP-ribose) polymerase in vitro and suppress the proliferation of human cells from leukemia, brain, breast, and colon cancers.[18] Several *C*-nitroso-based drugs have undergone preclinical studies, and some molecules are now engaged in clinical trials.[17,19]

Since it is well established that photo-triggered disassembly of NB molecules results in the production of aromatic *C*-nitroso compounds, we conceived that micelles made of NB-based amphiphiles could be used as photo-responsive nanoparticles to inhibit cell proliferation. To this end, amphiphilic molecules were designed by taking three important aspects into consideration: i) the ability of the molecules to self-assemble in aqueous media, ii) the biocompatibility of the final supra-molecular object through the use of a proper coating, iii) the light-induced release of cytotoxic nitroso derivatives. In previous studies, PEGylated amphiphiles were shown to spontaneously form biocompatible

Dr. P. Anilkumar, Dr. E. Gravel, Dr. E. Doris
CEA, iBiTecS, Service de Chimie
Bioorganique et de Marquage
91191, Gif-sur-Yvette, France
E-mail: eric.doris@cea.fr
I. Theodorou, Dr. F. Ducongé
CEA, I²BM, Molecular Imaging Research Center
(MIRCen), 18 route du panorama, BP n°6
F-92265, Fontenay-aux-Roses, France
E-mail: frederic.duconge@cea.fr
I. Theodorou, Dr. F. Ducongé
CNRS, Unité de Recherche Associée CEA-CNRS2210,
18 route du panorama, BP n°6
F-92265, Fontenay-aux-Roses, France
I. Theodorou, Dr. F. Ducongé, K. Gombert, B. Thézé
CEA, I²BM, Service Hospitalier Frédéric Joliot (SHFJ)
4 place du général Leclerc
91401, Orsay, France
I. Theodorou, Dr. F. Ducongé, K. Gombert, B. Thézé
INSERM U1023
Laboratoire d'Imagerie Moléculaire Expérimentale
4 place du général Leclerc
91401, Orsay, France
I. Theodorou, Dr. F. Ducongé, K. Gombert, B. Thézé
Université Paris Sud
4 place du général Leclerc
91401, Orsay, France

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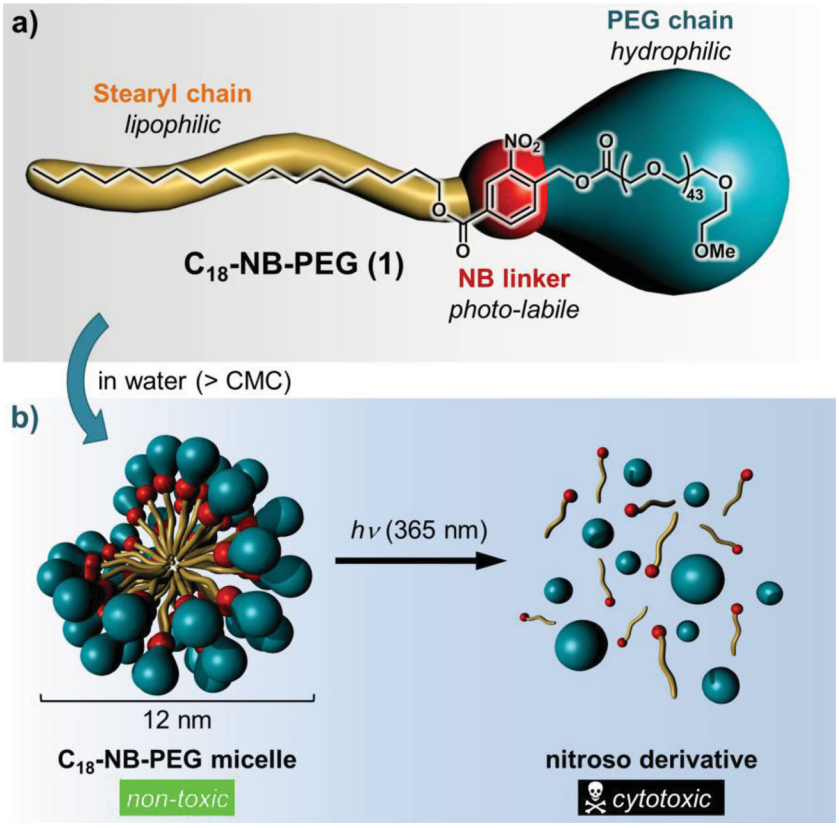


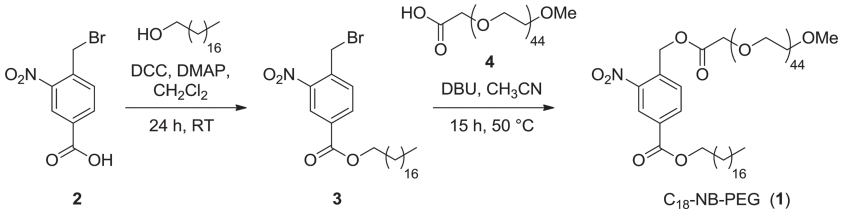
Figure 1. a) Structure of C_{18}-NB-PEG amphiphile (**1**), and b) formation of C_{18}-NB-PEG micelles and disruption of the latter under photo-irradiation to yield cytotoxic species.

nanosized micelles that were readily internalized into cells.[20] Here, a micelle-forming NB-based amphiphile (C_{18}-NB-PEG, **1**) was synthesized by linking a lipophilic stearyl chain and a hydrophilic PEG (M_w ca. 2000 g mol^{-1}) chain through a photo-labile NB-ester linkage. In brief, the C_{18}-NB-PEG amphiphile is expected to form stable micelles with light-triggered cytotoxic effect. The self-assembling properties of this new amphiphile and the photo-labile nature of the resulting micelles were investigated (**Figure 1**). In addition, the cellular uptake and the cytotoxic effect of the photo-product were evaluated in vitro on human breast cancer cells (MDA-MB-231).

2. Results and Discussion

2.1. Synthesis of the Amphiphilic Unit

The synthesis of the photo-cleavable amphiphilic unit **1** was achieved starting from 3-nitro-4-(bromomethyl)benzoic acid (**2**), which was esterified with stearyl alcohol in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) and catalytic 4-dimethylaminopyridine (DMAP) (**Scheme 1**). The resulting bromo-ester **3** was then connected to the PEG chain by nucleophilic displacement of the bromine atom of **3** by carboxylic acid-terminated polyethylene glycol **4** (M_w ca. 2000 g mol^{-1}) under basic conditions (1,8-diazabicyclo[5.4.0]undec-7-ene, DBU). The coupling reaction provided access to the target photo-cleavable amphiphilic unit which was thereafter self-assembled into micelles in aqueous medium.



Scheme 1. Synthesis of C_{18}-NB-PEG (**1**).

2.2. Micelle Assembly

The newly synthesized C_{18}-NB-PEG (**1**) was soluble in water upon gentle shaking and formed a foamy solution. To investigate the micellar behavior of amphiphile **1**, critical micelle concentration (CMC) measurements and dynamic light scattering (DLS) studies were carried out. The critical micelle concentration was assessed using the pyrene encapsulation technique.[21] A series of C_{18}-NB-PEG solutions were prepared at variable concentrations (from 0.005 to 1 g L^{-1}) in water containing pyrene (10^{-6} M), and fluorescence was measured. The ratio of the pyrene fluorescence intensity at wavelengths of 374 and 383 nm followed a non-linear trend when the concentration of C_{18}-NB-PEG was increased, indicating a micro-environmental change in solution (see **Figure 2**a). The inflexion point of the curve reflects the transition from monomers to micelles and occurs here at a concentration of 85 mg L^{-1}. This value corresponds to the critical micelle concentration that is the concentration above which the amphiphilic unimers self-assemble into micelles. To further characterize the micelles, a 10 mg mL^{-1} solution of **1** was subjected to DLS analysis revealing particles with a diameter of ca. 12 nm (Figure 2b). Static light scattering (SLS) experiments permitted to determine an apparent molecular weight of ca. 314 kg mol^{-1} which corresponds to ca. 125 amphiphiles per C_{18}-NB-PEG micelle. These morphological studies confirmed that **1** spontaneously formed nanometer-sized micelles in an aqueous medium.

2.3. Photophysical Study

Micelles generated by the assembly of C_{18}-NB-PEG (**1**) incorporate a photo-cleavable linkage connecting the hydrophobic core to the hydrophilic PEG. Upon photo-irradiation, bond cleavage occurs at the benzylic position resulting in the formation of a

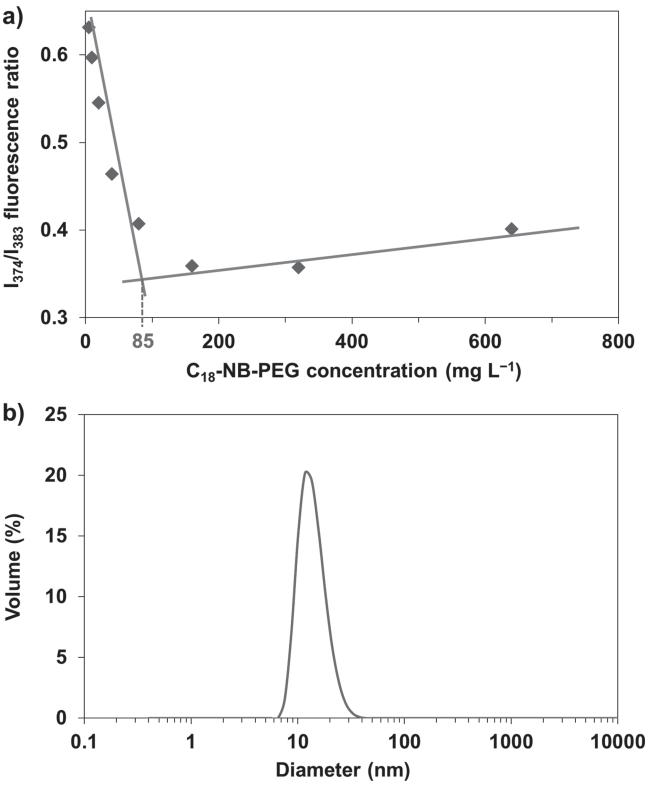


Figure 2. Micellar properties of C_{18}-NB-PEG. a) Plot of CMC determination, and b) size distribution of micelles by DLS.

nitrosobenzaldehyde derivative and a free PEG-carboxylic acid (**Scheme 2**).[22] As this process involves a rearrangement of the aromatic π-electrons, it can be detected through a change of the optical properties of the released product.[23] The kinetics of the photo-cleavage was thus monitored using absorption spectroscopy (**Figure 3**). Micelles made of C_{18}-NB-PEG showed a major absorption peak around 230 nm, a shoulder at 265 nm that corresponds to the electronic transition of the NB unit, and some minor absorption up to ca. 400 nm. For irradiation experiments we selected a longer ultraviolet wavelength at 365 nm because it is less damaging to cells than shorter UV. It can be seen from Figure 3 that after 5 min of irradiation, the intensity of the peak at 230 nm gradually decreased with the concomitant appearance of a new absorption band around 325 nm. This new band suggests the formation of a more delocalized aromatic system, which is the nitrosoaldehyde photo-product (see Scheme 2) in accordance with the literature.[24] As the time of light exposure increased, the peak at 230 nm decreased considerably and the intensity of the new band at 325 nm reached a maximum intensity after 120 min of irradiation. Absorption studies thus clearly demonstrated that upon UV irradiation at 365 nm C_{18}-NB-PEG undergoes cleavage, which should, in turn, disrupt the micelles.

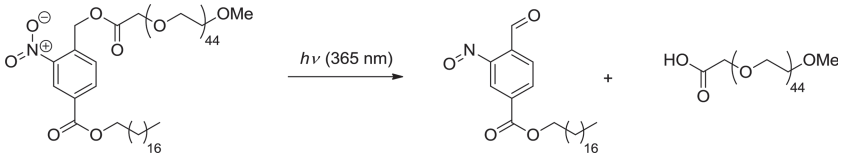
2.4. Photo-Disruption of the Micelle

To demonstrate the disruption of the micelles, the release of a payload upon photo-irradiation was investigated. Micelles were thus loaded with a dye by magnetically stirring Nile Red (10^{-6} M) with C_{18}-NB-PEG (**1**) in water (150 mg L^{-1}) for 2 h. The resulting mixture was kept undisturbed for 5 h to equilibrate the process. Fluorescence spectroscopy of the Nile Red-loaded micelles showed strong 633 nm emission upon excitation at 550 nm (**Figure 4**) as the dye is highly soluble and emissive in hydrophobic environments. However, Nile Red becomes less responsive with a spectral redshift upon release into aqueous medium.[25] This property of the dye was used to qualitatively demonstrate the photo-labile nature of the C_{18}-NB-PEG micelles. The dye-loaded micelles were subjected to photo-disruption at 365 nm and changes in the fluorescence emission of Nile Red were monitored as a function of time. After 30 min of irradiation, the fluorescence emission of the dye-loaded micelles showed a 1.5-time decrease in intensity. After 120 min of irradiation, a 3.5-time decrease in the emission intensity was detected with a 6 nm redshift, suggesting the release of Nile Red into water resulting from the photo-triggered cleavage of the micelles. As a control experiment, Nile Red dissolved in a 2:1 THF/water mixture was irradiated at 365 nm under the same conditions, but no modification of the fluorescence spectrum was observed.

Taken together, the above studies indicate that C_{18}-NB-PEG (**1**) readily formed micelles whose degradation could be triggered by light to produce cytotoxic nitroso derivatives. It was thus decided to explore the potential of the nitroso species as photo-triggered therapeutics by carrying out a series of in vitro studies.

2.5. Cellular Uptake of the Micelles

The first in vitro aspect that we investigated was the ability of the C_{18}-NB-PEG micelles to be internalized into cells. In previous studies, pegylated nanometer-sized micelles were shown to be biocompatible and readily uptaken by human cancer cells.[20,26] To confirm that C_{18}-NB-PEG micelles show a similar behavior, they were loaded with 5 wt% of fuorescent DiO dye (λ_{em} = 501 nm) prior to incubation with MDA-MB-231 cancer cells. The use of DiO as label made the micelles suitable for fluorescent microscopy experiments allowing assessment of cellular internalization.[26] The DiO-labeled micelles were incubated with MDA-MB-231 cells at a concentration of 800 nM for 12 h (**Figure 5**). Then, the cells were washed thoroughly and observed by wide field epi-fluorescence microscopy. A fluorescence signal was detected within intracellular vesicles, which demonstrated the ability of the C_{18}-NB-PEG micelles to be internalized into MDA-MB-231 cells. To confirm that the observed fluorescence was indeed associated with the micelles, a control



Scheme 2. Products formed from C_{18}-NB-PEG upon photolysis.

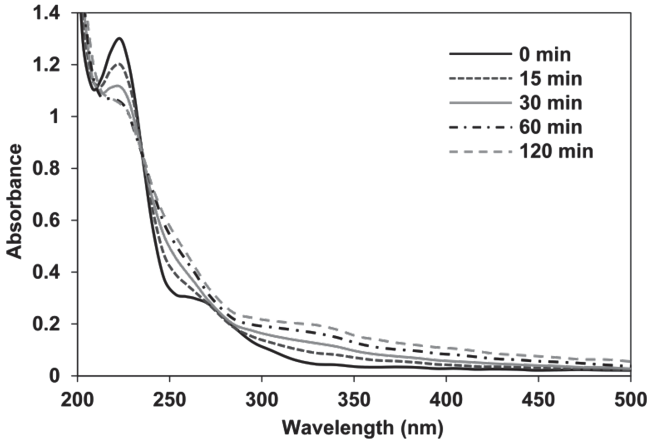


Figure 3. Absorption profile of C_{18}-NB-PEG micelles after 0, 5, 15, 60, and 120 min of irradiation at 365 nm.

experiment was conducted without micelles showing no intracellular signal. As the cellular uptake of the micelles was confirmed, the next step was to investigate their photo-triggered cytotoxicity on cancer cells.

2.6. In vitro Cytotoxicity Studies

In vitro cytotoxic assays were conducted on MDA-MB-231 cancer cells as a model system. First, a control experiment without micelles was set up by simply irradiating the cells at 365 nm for 1 h in phosphate buffered saline (PBS). In this case, no cell death was observed, thus confirming the non-lethality of the operating conditions. The same experimental conditions were hence used for the subsequent irradiations, but in presence of C_{18}-NB-PEG micelles. In order to get a clear picture of the cell proliferation inhibition, three sets of experiments were designed: i) C_{18}-NB-PEG micelles were incubated with cells without irradiation; ii) micelles were pre-irradiated for 1 h before incubation with cells; and iii) micelles were

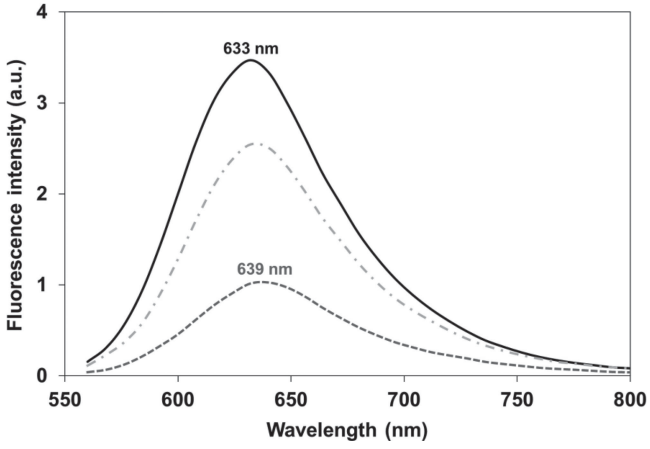


Figure 4. Fluorescence profile (excitation 550 nm) of Nile Red encapsulated inside the C_{18}-NB-PEG micelles before irradiation (—), and after 30 min (· · · ·) or 120 min (– – –) of photo-irradiation at 365 nm.

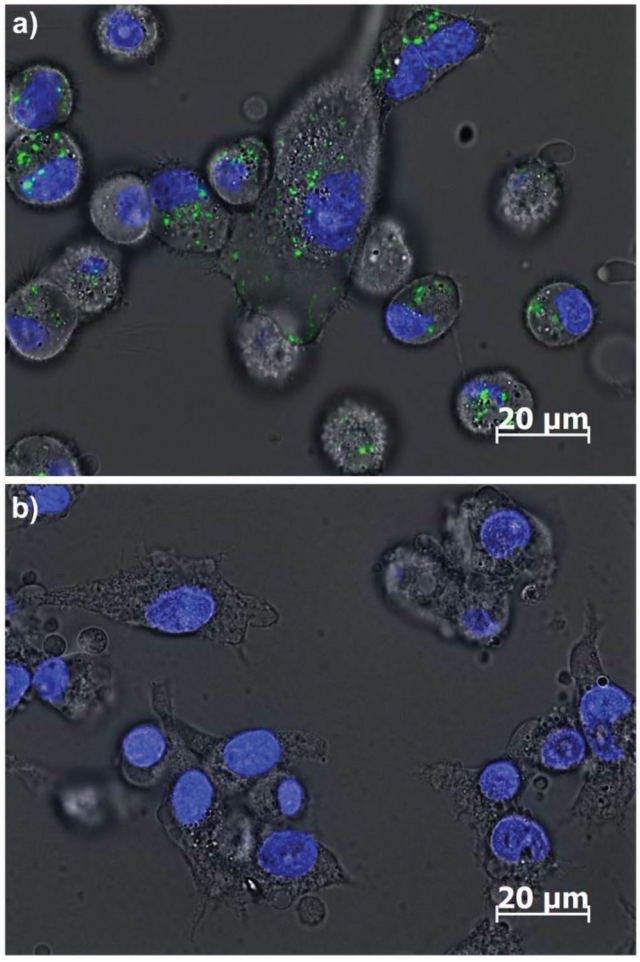


Figure 5. Widefield micrographs obtained after incubating MDA-MB-231 cells for 12 h either a) with C_{18}-NB-PEG micelles or b) without micelles. The images show the widefield image merged with the fluorescence signals of Hoechst dye (blue, nuclei) and DiO (green, micelles).

first incubated with cells and then subjected to irradiation for 1 h. In each case, cells were incubated with varying concentrations of the micelles (from 0.2 to 60 µM) for 12 h, and the cell viability and proliferation was assessed using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) colorimetric test (**Figure 6**).

In the first set of experiments in which the micelles were not irradiated, no significant cell death was observed in comparison to untreated cells, regardless of the micelle concentration. This result indicates that there is no intrinsic toxicity associated with our C_{18}-NB-PEG micellar system in the absence of irradiation. In the second experiment, in which the micelles were irradiated prior to incubation, there was no cytotoxic effect until the micelle concentration reached 6 µM. Above this value, a slight effect was detected in the 30–60 µM range with an IC_{50} value of 38 µM. This result suggests a lethal effect of the photo-produced nitrosobenzaldehyde derivative at moderately high concentrations. More interestingly, when micelles were irradiated after incubation with cells (third set of experiments), a more drastic effect was observed. At very low concentration (0.2 µM) the irradiated micelles showed no toxicity, but as the concentration

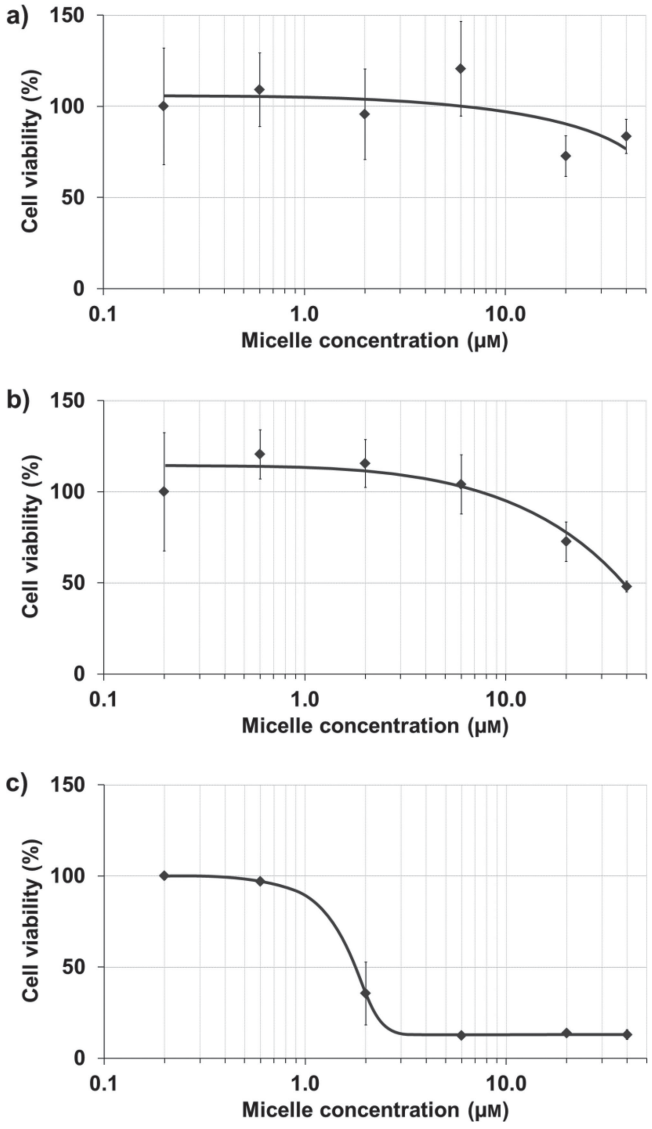


Figure 6. MTT assay results for C_{18}-NB-PEG micelles which were incubated with MDA-MB-231 cells either a) without irradiation, b) after irradiation, or c) before irradiation.

gradually rose, toxicity became significant causing the death of almost 64% of the cells at 2 μM and nearly 90% at 6 μM. The IC_{50} calculated from the latter experiments was 1.7 μM. As a comparison, when paclitaxel in its commercial formulation (Taxol) was used under the same conditions, results indicated an IC_{50} of 15 μM, with or without UV irradiation after incubation with cells.

The absence of any cellular toxicity when micelles were not irradiated compared to the toxicity of micelles that had undergone irradiation clearly demonstrates that cell death is caused by the photo degradation products of the micelles. However, there is a significant difference in the cytotoxicity of C_{18}-NB-PEG micelles depending on whether they were irradiated before or after incubation with the cells. We hypothesize that this difference in the inhibition of the cellular proliferation is related to variable uptake by the cells. In the former case (irradiation prior to incubation), micelles were disrupted before cellular interaction had taken place, leaving free nitrosobenzaldehyde derivatives to be internalized by the cells or to interact with extracellular components such as serum proteins. In contrast, when irradiation of the micelles was performed after incubation with cells, the photo-disruption process takes place in the intracellular space, leading to the release of cytotoxic nitrosobenzaldehyde inside of the cells. In the latter case, two different mechanisms could account for the observed cytotoxicity: i) delivery of a higher intracellular concentration of the nitroso compound and/or ii) delivery of the active compound in different cellular compartments. Whatever the mechanism, the generation of the nitrosobenzaldehyde from the internalized micelles affects the inhibition of cellular proliferation far more drastically.

3. Conclusions

A photo-labile C_{18}-NB-PEG amphiphile was synthesized by connecting stearyl and PEG-2000 chains via a nitrobenzyl ester linkage. This compound readily formed micelles of 12 nm diameter that underwent photo-cleavage upon UV irradiation at 365 nm, resulting in the formation of a cytotoxic nitrosobenzaldehyde derivative. In vitro studies validated the potential of C_{18}-NB-PEG micelles for the on-demand inhibition of cell proliferation by a photo-triggered cytotoxic effect. In addition to light-induced cytotoxicity, a higher toxicity in cancer cells compared to healthy cells may be further achieved by conjugating the micelles with a targeting moiety to enhance selective cellular internalization. Moreover, as C_{18}-NB-PEG micelles can also be loaded with hydrophobic compounds,[27] the inclusion of drugs in the core of the carrier could potentiate its cytotoxic effect by the synergistic photo-release of the payload and generation of the nitroso derivative. The results obtained in the context of this study open the way to future directions such as the in vivo evaluation of the photo-responsive micellar system using near infrared two-photon excitation, which would benefit from deeper diffusion and lower tissue damage.[28]

4. Experimental Section

4.1. Materials and Methods

Polyethylene glycol monomethyl ether (M_w ca. 2000 g mol^{-1}), DCC, sodium hydride, 2-bromoacetic acid, 3-nitro-4-(bromomethyl)benzoic acid, stearyl alcohol, DMAP, DBU, Nile Red, and pyrene were purchased from Sigma-Aldrich and used without further purification. Solvents were purchased locally and purified following standard protocols before use. NMR analysis was performed using a Bruker Avance NMR spectrometer operating at 400 MHz (^{1}H) and 100 MHz (^{13}C). DLS measurements were carried out using a Malvern Zetasizer instrument employing a 4 mW He–Ne laser (λ = 632.8 nm) and equipped with a thermostatic sample chamber. UV-vis spectra were recorded using a Cary 50 Probe spectrophotometer. Fluorescence spectra were recorded on a Horiba Jobin Yvon Fluoromax-4 spectrometer. Photo-irradiation experiments were carried out using a long wave 100 W UV lamp with an inbuilt filter (Blak-Ray) emitting light of 365 nm wavelength at an intensity of approx. 15 mW cm^{-2} (10 cm distance). Culture reagents were purchased from Life Technologies.

4.2. Synthesis of Compound 2

N,N'-dicyclohexylcarbodiimide (396 mg, 1 equiv.) in CH_2Cl_2 (2.5 mL) was added to a mixture of 3-nitro-4-(bromomethyl)benzoic acid (500 mg, 2 mmol, 1 equiv.), stearyl alcohol (520 mg, 1 equiv.), and DMAP (20 mg, cat.) in CH_2Cl_2 (10 mL) under nitrogen at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and 24 h at room temperature. The precipitate was separated by filtration over paper and the crude product was recovered by evaporation of the solvent. The product was further purified by silica gel column chromatography (hexane/EtOAc, 90:10) to yield a pale yellow solid (820 mg, 80%). ^{1}H NMR ($CDCl_3$) δ: 8.65 (s, 1H), 8.25 (d, J = 8 Hz, 1H), 7.67 (d, J = 8 Hz, 1H), 4.85 (s, 2H), 4.37 (t, J = 7 Hz, 2H), 1.42–1.19 (m, 32H), 0.88 (t, J = 6.7 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ: 163.9, 147.9, 136.8, 134.1, 132.7, 132.1, 126.4, 66.1, 31.8, 29.6, 29.5, 29.4, 29.3, 29.2 27.9, 25.8, 22.6, 14.1.

4.3. Synthesis of Compound 4

Under nitrogen, a suspension of washed sodium hydride (60 mg, 5 equiv.) in THF (10 mL) was added to a solution of PEG-2000 monomethylether (1 g, 0.5 mmol, 1 equiv.) in THF (10 mL). The resulting mixture was refluxed for 30 min and brought down to room temperature. 2-bromoacetic acid (140 mg, 2 equiv.) dissolved in THF (5 mL) was then added dropwise over a period of 30 min and the mixture was subsequently stirred for 40 h at room temperature. The reaction was quenched by addition of 1 M HCl (10 mL), and the mixture was extracted with CH_2Cl_2 (3 × 10 mL). The organic layers were pooled, dried over $MgSO_4$, filtered and concentrated under vacuum. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH, 85:15) to yield a white solid (770 mg, 75%). ^{1}H NMR ($CDCl_3$) δ: 4.15 (s, 2H), 3.63 (m, 176H), 3.37 (s, 3H); ^{13}C NMR ($CDCl_3$) δ: 171.6, 71.8, 70.9 (multiple C), 70.5, 68.8, 58.9.

4.4. Synthesis of C_{18}-NB-PEG (1)

Under nitrogen, DBU (66 mg, 0.4 mmol, 1 equiv.) in MeCN (5 mL) was added dropwise to a mixture of compound **2** (280 mg, 1.2 equiv.) and PEG **4** (900 mg, 1 equiv.) in CH_3CN (10 mL). The reaction was heated to 50 °C for 15 h. The solvent was then evaporated and the crude product was purified by silica gel column chromatography (CH_2Cl_2/MeOH 90:10) to yield a pale yellow solid (700 mg, 70%). ^{1}H NMR ($CDCl_3$) δ: 8.73 (s, 1H), 8.29 (d, J = 8.1 Hz, 1H), 7.70 (d, J = 8.1 Hz, 1H), 5.64 (s, 2H), 4.37 (t, J = 6.7 Hz, 2H), 4.29 (s, 2H), 3.66 (m,176H), 3.38 (s, 3H), 1.42–1.18 (m, 32H), 0.88 (t, J = 7 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ: 169.7, 164.1, 147.2, 136.1, 134.2,131.5, 129.1, 126.1, 71.8, 71.1, 70.4 (multiple C), 68.4, 66.1, 62.8, 58.9, 54.5, 48.7, 37.9, 32.3, 31.8, 29.6, 29.4, 29.3, 29.2, 28.9, 28.5, 26.8, 25.8, 23.9, 22.6, 19.1, 14.1.

4.5. Determination of the Critical Micelle Concentration

The CMC of C_{18}-NB-PEG (**1**) was determined following the pyrene encapsulation method. In a typical experiment, C_{18}-NB-PEG solutions of varying concentrations (from 0.005 to 1 g L^{-1}) were used to dissolve pyrene (10^{-6} M) by sonication. The obtained colloidal solution was kept undisturbed for 5 h, then fluorescence spectra (excitation at 339 nm) were recorded and the ratio of fluorescence intensity at 374 and 383 nm (I_{374}/I_{383}) was measured and plotted against the amphiphile concentration. The CMC value was obtained at the intersection of the two lines and was found to be 85 mg L^{-1}.

4.6. Photo-Irradiation Experiments

10 mg of C_{18}-NB-PEG (**1**) was dissolved in deionized water by gentle shaking at room temperature. The resulting micelle solution was filtered using a 0.22 µm sized membrane filter and the solution was kept undisturbed for 5 h. In order to study the changes in absorption upon exposure to UV light, the solution was irradiated at 365 nm. The sample cuvette was kept at a distance of 10 cm from the lamp. The absorption spectrum of the sample was recorded at various time intervals.

To confirm the photo release properties of C_{18}-NB-PEG micelles, a solution was prepared by stirring the amphiphile (150 mg L^{-1}) in water containing Nile Red dye (10^{-6} M) for 2 h. The resulting Nile Red-loaded micelles were kept undisturbed for 5 h and filtered using a 0.22 µm sized membrane to remove any aggregates. The micelles were then irradiated at 365 nm and the corresponding changes in the fluorescence of Nile Red were monitored by fluorescence spectroscopy at various time intervals.

4.7. Dynamic Light Scattering and Static Light Scattering

10 mg of C_{18}-NB-PEG (**1**) was dissolved in deionized water by gentle shaking at room temperature. The resulting micelle solution was filtered using a 0.22 µm sized membrane filter and kept undisturbed for 5 h.

Size measurements were carried out using a Nano ZS Malvern instrument in DLS mode over 12 runs of 10 s each.

Apparent molecular weight measurements were carried out using the same instrument in SLS mode with water as standard and micelle concentrations ranging from 0.1 to 1 mg mL^{-1}.

4.8. Cellular Uptake

DiO (500 µg) solubilized in chloroform (50 µL) was added to a 10 mg mL^{-1} colloid suspension of C_{18}-NB-PEG micelles (1 mL) and the mixture was sonicated (ultrasonic probe, 300 ms pulses per second, 25 W output power) for 3 × 10 min. The clear orange colloid was filtered through a 0.22 µm membrane and stored in the dark at 4 °C. 48 h before the experiment, 20×10^3 MDA-MB-231 cells (human breast carcinoma; ATCC) were seeded on a collagen-coated, 4-well microscope slide (Labteck). Cells were grown in DMEM medium with phenol red (Gibco), 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% antibiotic antimycotic solution (Sigma–Aldrich).

DiO-loaded C_{18}-NB-PEG micelles were incubated for 12 h with the cells at a final working concentration of 800 nM in DMEM medium (with phenol red, 10% FBS and 1% antibiotic antimycotic solution).

Prior to microscopy, cells were washed (3 times) and imaged in D-PBS with Hoechst 33342 (Life Technologies) at 2.5 µg mL^{-1}.

Images were obtained using a wide field AxioObserver Z1 epi-fluorescence microscope (Zeiss, Germany) with a 63× objective (N.A. 1.40).

4.9. Cytotoxicity Assessment

Human breast cancer MDA-MB-231 cells (human breast carcinoma from ATCC) were grown in DMEM medium with phenol red (Gibco), 10% FBS (Sigma-Aldrich) and 1% antibiotic antimycotic solution (Sigma–Aldrich). In a 96 well plate, 2×10^3 cells diluted in 50 µL of culture medium were seeded per well. After 24 h in a cell incubator, 50 µL of D-PBS containing the micelles at different concentrations (0.2, 0.6, 2, 6, 20, 40 µM) was added. The plate was then allowed to stand in the cell incubator for 12 h and the medium was replaced with D-PBS before irradiation, after which D-PBS was replaced again with culture medium. The plate was then allowed to stand in the cell incubator for 24 h before adding 20 µL of methylthiazolyldiphenyl-tetrazolium bromide (MTT). The plate was analyzed with a Mithras microplate reader (LB 940, Berthold) at 490 nm, 2 h after incubation with MTT in the cell incubator. The data were compared to cells that underwent the same procedure but without micelles and irradiation. The experiments were performed in triplicate. The same procedure was followed using cells without addition of micelles or without irradiation but using pre-irradiated micelles. The pre-irradiation of micelles was performed under the same conditions as the irradiation of cells previously described. The plot was expressed as a

function of the percentage of living cells, 100% being the well containing only cells and MTT.

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[1] D. Peer, J. M. Karp, S. Hong, O. C. Farokhzad, R. Margalit, R. Langer, *Nat. Nanotechnol.* **2007**, *2*, 751.
[2] M. L. Immordino, F. Dosio, L. Cattel, *Int. J. Nanomed.* **2006**, *1*, 297.
[3] J. Zhua, X. Shi, *J. Mater. Chem. B* **2013**, *1*, 4199.
[4] a) R. Duncan, *Nat. Rev. Drug Discovery* **2005**, *2*, 347; b) R. Haag, F. Kratz, *Angew. Chem. Int. Ed.* **2006**, *45*, 1198.
[5] M. E. Davis, Z. Chen, D. M. Shin, *Nat. Rev. Drug Discovery* **2008**, *7*, 771.
[6] A. N. Lukyanov, V. P. Torchilin, *Adv. Drug Delivery Rev.* **2004**, *56*, 1273.
[7] V. P. Torchilin, *Curr. Drug Delivery* **2005**, *2*, 319.
[8] R. Trivedi, U. B. Kompella, *Nanomedicine* **2010**, *5*, 485.
[9] a) J. M. Harris, R. B. Chess, *Nat. Rev. Drug Discovery* **2003**, *2*, 214; b) L. E. van Vlerken, T. K. Vyas, M. M. Amiji, *Pharm. Res.* **2007**, *24*, 1405.
[10] G. Mayer, A. Heckel, *Angew. Chem. Int. Ed.* **2006**, *45*, 4900.
[11] J.-F. Gohy, Y. Zhao, *Chem. Soc. Rev.* **2013**, *42*, 7117.
[12] I. Aujard, C. Benbrahim, M. Gouget, O. Ruel, J-.B. Baudin, P. Neveu, L. Jullien, *Chem. Eur. J.* **2006**, *12*, 6865.
[13] A. M. Kloxin, A. M. Kasko, C. N. Salinas, K. S. Anseth, *Science* **2009**, *324*, 59.
[14] J. Jiang, X. Tong, D. Morris, Y. Zhao, *Macromolecules* **2006**, *39*, 4633.
[15] S. S. Agasti, A. Chompoosor, C.-C. You, P. Ghosh, C. K. Kim, V. M. Rotello, *J. Am. Chem. Soc.* **2009**, *131*, 5728.
[16] A. Blanc, C. G. Bochet, *J. Am. Chem. Soc.* **2004**, *126*, 7174.
[17] X. Liu, Y. Shi, D. X. Maag, J. P. Palma, M. J. Patterson, P. A. Ellis, B. W. Surber, D. B. Ready, N. B. Soni, U. S. Ladror, A. J. Xu, R. Iyer, J. E. Harlan, L. R. Solomon, C. K. Donawho, T. D. Penning, E. F. Johnson, A. R. Shoemaker, *Clin. Cancer Res.* **2012**, *18*, 510.
[18] a) W. G. Rice, C. D. Hillyer, B. Harten, C. A. Schaeffer, M. Dorminy, D. A. LackeyIII, E. Kristen, J. Mendeleyev, K. G. Buki, A. Hakam, E. Kun, *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 7703; b) K. G. Buki, P. I. Bauer, J. Mendeleyev, A. Hakam, E. Kun, *FEBS Lett.* **1991**, *290*, 181; c) J. Mendeleyev, E. Kristen, A. Hakam, K. G. Buki, E. Kun, *Biochem. Pharm.* **1995**, *50*, 705.
[19] J. O'Shaughnessy, C. Osborne, J. E. Pippen, M. Yoffe, D. Patt, C. Rocha, I. C. Koo, B. M. Sherman, C. Bradley, *N. Engl. J. Med.* **2011**, *364*, 205.
[20] a) E. Gravel, J. Ogier, T. Arnauld, N. Mackiewicz, F. Ducongé, E. Doris, *Chem. Eur. J.* **2012**, *18*, 400; b) N. Mackiewicz, E. Gravel, A. Garofalakis, J. Ogier, J. John, D. M. Dupont, K. Gombert, B. Tavitian, E. Doris, F. Ducongé, *Small* **2011**, *7*, 2786.
[21] A. Domínguez, A. Fernández, N. González, E. Iglesias, L. Montenegro, *J. Chem. Educ.* **1997**, *74*, 1227.
[22] H. Zhao, E. S. Sterner, E. B. Coughlin, P. Theato, *Macromolecules* **2012**, *45*, 1723.
[23] B. Chandra, R. Subramaniam, S. Mallik, D. K. Srivastava, *Org. Biomol. Chem.* **2006**, *4*, 1730.
[24] Y. V. Il'ichev, M. A. Schwörer, J. Wirz, *J. Am. Chem. Soc.* **2004**, *126*, 4581.
[25] J. Jiang, X. Tong, D. Morris, Y. Zhao, *Macromolecules* **2006**, *39*, 4633.
[26] E. Gravel, B. Thézé, I. Jacques, P. Anilkumar, K. Gombert, F. Ducongé, E. Doris, *Nanoscale* **2013**, *5*, 1955.
[27] J. Ogier, T. Arnauld, G. Carrot, A. Lhumeau, J.-M. Delbos, C. Boursier, O. Loreau, F. Lefoulon, E. Doris, *Org. Biomol. Chem.* **2010**, *8*, 3902.
[28] a) I. Aujard, C. Benbrahim, M. Gouget, O. Ruel, J. B. Baudin, P. Neveu, L. Jullien, *Chem. Eur. J.* **2006**, *12*, 6865; b) J. Cao, S. Huang, Y. Chen, S. Li, X. Li, D. Deng, Z. Qian, L. Tang, Y. Gu, *Biomaterials* **2013**, *34*, 6272.