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Near Infrared Light-Triggered Drug Generation and Release from Gold Nanoparticle Carriers for Photodynamic Therapy

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Abstract

A photoprecursor Pc 227 is covalently bound onto gold nanoparticles (Au NPs) to produce the known photodynamic therapy (PDT) drug Pc 4 upon 660 nm photoirradiation. The photochemical formation of the photoproduct Pc 4 is identified by spectroscopy, chromatography, and mass spectrometry and its PDT efficacy is equal to Pc 4 when administered non-covalently by Au NPs, with the added benefit of improved covalent delivery and targeted NIR-triggered release from the covalent Pc 227-Au NP conjugate, while during transport the attached Pc 227 is quenched by the Au NP and PDT inactivated.

Controlled delivery of the typically hydrophobic drugs is one of the major challenges encountered in photodynamic therapy (PDT) cancer treatment. PEGylated gold nanoparticle (Au NP)-based delivery systems have attracted significant attention to improve the solubility and selectivity of PDT drugs due to their properties of biocompatibility, high drug loading capacity, chemically versatile surface, and controlled drug release. [1,2] As an external stimulus, light is widely employed for controlled drug release in the treatment of diseases. [3–6] However, most of the photo-stimulated drug delivery systems utilize ultraviolet light below 400 nm to release the drug molecules. [7–10] Unfortunately, short-wavelength light has poor skin penetration and can cause the damage to DNA which limits their application. In contrast, the visible-near infrared (Vis-NIR) range of light between 620 nm and 850 nm, the so-called “phototherapeutic window”, is harmless with maximum tissue penetration. [11,12] A Vis-NIR-triggered drug release holds promise for applications in drug delivery and phototherapy. [13,14]

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The use of silicon phthalocyanine 4 (**Pc 4**) as a hydro-phobic PDT drug has been developed and is currently FDA-approved in clinical trials. [15,16] A PEGylated Au NP based delivery system with improved water solubility and stability has been shown to greatly enhance the efficacy of **Pc 4** in vivo through non-covalent drug delivery. [17,18] Although covalent delivery provides a stable delivery approach, it is a formidable challenge to control the drug release with a stimulus while maintaining the therapeutic and biological properties of the drug load. In some instances, covalent attachment of drugs to the vector can alter the delivery mechanism and even eliminate any PDT efficacy, as was recently observed in HeLa cells. [17]

In this communication, we describe an example of a Vis-NIR-light triggered PDT drug delivery-and-release system by attaching **Pc 227** (a **Pc 4 precursor**) on a Au NP via a Au-S bond. **Pc 227** is designed with a NIR photosensitive Si-C bond (**Scheme 1**, red bond) in order to enable the drug release through 660 nm Vis-NIR irradiation. It is known that photolysis of Si-C bonds on axial ligands of silicon porphyrin complexes undergoes homolysis during visible light irradiation. [20,21] Phthalocyanine-based photosensitizers used herein have strong absorptions with $\varepsilon_{max} = 2 \times 10^5$ cm $^{-1}$ M^{-1} in the 660–690 nm wavelength regime. [22] Similar to the published silicon porphyrin complexes, the axial alkyl ligand on silicon phthalocyanines is photosensitive and can photocleave the Si-C bond homolytically. [21] To the best of our knowledge, this is the first report of a NIR photo-cleavable drug delivery system, which results in a fully active and FDA-approved PDT agent (**Pc 4**) upon release.

Here, we describe the synthesis, characterization, and Vis-NIR light activation of this novel conjugate, and demonstrate the PDT efficacy in vitro. The synthesis of **Pc 227** was previously published and confirmed using both NMR and MS analysis. [19] Thus it is simple to differentiate between **Pc 227** and **Pc 4** . [15] On the other hand, the photophysical properties of **Pc 227** are very similar to those of **Pc 4** and generally reflect the main contributions of the aromatic macrocyclic ring structure with minor electronic effects from the axial ligands. From the absorption (**Figure 1A**) and fluorescence (Figure 1B) of **Pc 227** and **Pc 4** , it is apparent that **Pc 227** absorbs and emits light at slightly longer wavelengths compared to **Pc 4,** which made it straightforward to follow the progress of the photolysis reaction by monitoring the resulting Vis-NIR spectra. Thus, the conversion of **Pc 227** to the photoactive **Pc 4** could be monitored (Figure 1C) and additionally confirmed by UV-Vis and fluorescence spectrometry (Figure 1E and 1F). The photolysis of **Pc 227** in methanol was monitored over time at the laser wavelength of 660 nm with the power of 0.082 mW. Changes in the absorption and fluorescence spectra were recorded at different intervals during the irradiation. 10 seconds post of the irradiation, the Q band of **Pc 227** at 676 nm shifted to 670 nm, which was the same as **Pc 4** . The blue shifted of the Q band was completed at 180 sections post of the irradiation. There was no significant change post 4 minutes and 5 minutes of irradiation. A similar blue shift trend in the emission of the photolysis product was observed in the fluorescence spectra. Compared to the **Pc 227** , the maximum emission of the photolysis product showed a 7 nm blue shift. The fluorescence quantum yield of **Pc 227** was 1% and **Pc 4** was 16.9% using methylene blue as the reference. The increased fluorescence intensity upon irradiation confirmed the generation of

Pc 4 . These results suggest that the photolysis of **Pc 227** is efficient and could generate **Pc 4** upon irradiation.

Singlet oxygen $^1O_2$ is the reactive species in PDT and highly toxic to the biomolecules. [16] It can be generated by energy transfer from the excited photosensitizer to the molecular oxygen. Silicon phthalocyanine molecules with high singlet oxygen generation yields are excellent photosensitizer for PDT. After photolysis of **Pc 227** , the singlet oxygen quantum yield (Φ_Δ %) was measured by the photodecomposition of 1,3-diphenylisobenzenofuran (DPBF) in methanol. Methylene blue (Φ_Δ = 49%) was the reference compound. The photolysis products of **Pc 227** showed similar singlet oxygen generation ability as **Pc 4** in methanol. 49.0±3.5% quantum yield of the products was observed.

To achieve the light sensitive Au NP- **Pc 227** conjugate, dodecylamine coated Au NPs were synthesized using the Brust-Schiffrin method and coated with MeO-PEG-SH (MW = 5000) in order to achieve solubility in water. [18] In order to achieve a stable conjugate in water, **Pc 227** with the thiol group on the axial ligand was conjugated on the Au NP and the final **Pc 227** molecules per NP was controlled to be 40 : 1 quantified via UV-Vis spectrometry. Transmission electron microscopy (TEM) revealed that the core size of the Au NP- **Pc 227** conjugates were ~ 5.5 nm, which was slightly smaller when compared to the dodecylamine coated Au NPs (**Figure 2**), indicating the etching via the thiol molecules. These conjugates were protected by the PEG with neural charge, which made them fairly stable in aqueous solutions for at least one year stored in dark. Successful incorporation of the caged **Pc 4** into the amphiphilic polyethylene glycol corona of the Au NPs (**Au NP-Pc 227**) was tested by assessing the photophysical properties of the NP conjugates in water. It is clear from the absorbance spectra in water (**Figure 3A**) that the drug was successfully conjugated to the NP. The Au NP surface plasmon band was at 520 nm, suggesting the Au NPs were well dispersed in water. In addition, the attached **Pc 227** showed the distinct band at 690 nm. However, the fluorescence of **Pc 227** was largely quenched in aqueous media (Figure 3B). When the conjugates are photolyzed with 660 nm light, the Q band at 690 nm blue-shifted, indicating successful photoloysis (Figure 3C). As can be expected, there is no significant formation of **Pc 4** fluorescence in aqueous solution due to the lack of solubility in water (Figure 3D). It is observed that in aqueous media the hydrophobic **Pc 4** does not diffuse away from the Au NP surface and thus remains quenched by the Au NP and its aqueous environment. This conclusion was further confirmed when the same experiment was conducted in organic media. In chloroform, the **Pc 4** absorption band at 673 nm increases with photolysis time while the original **Pc 227** peak at 690 nm decreases (Figure 3E).

Similar to free **Pc 227** , the photolysis products on the NPs show an increase in fluorescence intensity at 677 nm (Figure 3F). The **Au NP-Pc 227** behaves quite differently in aqueous versus lipophilic solvents. Although in both solvent environments drug conversion was observed (Figure 3C & E), only weak fluorescence of the photoproduct was observable in aqueous media (Figure 3D) when compared to the same experiment in organic solvent (Figure 3F), which is due to the hydrophobicity of **Pc 4** . The drug **Pc 4** can only be released from the NP in apolar environments (cell walls and organic solvents). On the other hand, in highly polar environments (blood stream and other aqueous media) the drug is hydro-

phobically forced into the Au NP interior where the photoex-cited state and its fluorescence are effectively quenched.

Importantly, **Pc 227** is similarly photolabile on the surface of Au NPs as the free molecule in solution. Upon irradiation, the Si-C bond of **Pc 227** is cleaved and generates a free valency on the central silicon. In aqueous solution, the unsaturated valence of the central silicon in the phthalocyanine ring reacts with H_2O and produces **Pc 4** (**Figure 4**) as a reaction product. The ESI-MS of **Au NP-Pc 227** conjugates after irradiation identified the generation of **Pc 4** at 717 m/z (Figure 4B). In addition, the major peaks at 701.9 m/z, 674.1 m/z, 577.6 m/z in Figure 4A were identified with the corresponding ion after the loss of a methyl group, the amine functionality and the whole upper axial ligand, respectively. The peak at 288.3 m/z was identified as an impurity from purging with just solvent (methanol). In keeping with previous reports of Au NP mediated **Pc 4** drug delivery, [18] the low water solubility of the phthalocyanine ring requires a non-polar media to mediate drug release. The use of the photolabile Si-C linker allows for Vis-NIR light triggered drug release from the Au NP surface.

PEGylated Au NPs as used in this study show little cellular uptake (~1%) and minimal toxicity to the cancer cells. [17,18] As shown in previous studies, the 24-hour incubation time is the optimal condition to reach the maximum intracellular drug concentration for PDT. [17,18] Therefore, the cancer cells were incubated with the **Au NP-Pc 227** conjugates for 24 hours and the cytotoxicity were evaluated by trypan blue exclusion staining and MTT assays, respectively. Trypan blue can penetrate the membrane of dead cells and stains those dead cells only. As shown in **Figure 5A**, most of the cells in the images are not stained by trypan blue, which indicates minimal toxicity of the **Au NP-Pc 227** at the performed incubation conditions in the dark. This was further confirmed with MTT assays (Figure 5B) to determine the cell survival after 24 hours incubation with the **Au NP-Pc 227** conjugates.

No toxicity was observed in the dark with a **Pc 227** concentration of 1μM when compared to the control samples. The photolysis product of **Pc 227** was **Pc 4** as confirmed through mass spectrometry measurements (Figure 4) and showed accordingly an equal singlet oxygen generation yield of 49.0 ± 3.5%, suggesting the efficient cytotoxicity could be induced via the irradiation. Although the effective release and generation of a **Pc 4** PDT agent from the **Au NP-Pc 227** conjugates was demonstrated in pure solvents, cell studies were required to determine whether the PDT efficacy of the phototriggered drug delivery system was preserved and could be used as an alternative to previously reported phthalocyanine PDT systems. [17,18] Clearly, the PDT effect of the conjugates in HeLa cell cultures was observed based on the change of cell morphology (Figure 5A). Before the PDT treatment, the cells were slightly elongated and dense monolayers could be observed after incubation with the **Au NP-Pc 227** conjugates. After irradiation, the **Au NP-Pc 227** treated HeLa cells changed to a round shape and showed shrinkage in size, indicating cell death. Additionally, the cells were stained with trypan blue after PDT treatment, and we could further confirm that the conjugates were effective PDT systems (Figure 5A). No cell was found alive at 1 μM **Au NP-Pc 227** concentration and 1 μJ light, and most of the dead cells were detached from the surface of the Petri dish after staining. To obtain a relative efficacy data, MTT assays were conducted to compare covalently attached **Au NP-Pc 227** conjugates to free **Pc 4 and Pc**

227 (Figure 5B). After light exposure, over 80% of the HeLa cells were killed when incubated with **Au NP-Pc 227** conjugates, which is close to the free **Pc 4** in DMF solution. In addition, cell studies showed that **Pc 227** alone was the least efficient reagent for PDT treatment compared to **Pc 4** and the **Au NP-Pc 227** conjugates, due to the fact that it first photolyzes.

We have previously shown that neutral Au NP conjugates have moderate interaction with the cell membranes, with subsequent release of the non-covalent drug through cell membrane diffusion. [17] Here, the photolysis of the covalent **Pc 227** -Au NP bond leads to release of the PDT agent near the cell surface, which is sufficiently close for cell uptake and effective PDT. As the active PDT drug **Pc 4** can be photogenerated from the attached **Pc 227** , the same cell uptake and PDT mechanism of the photoproduct **Pc 4** as previously reported are observed. [17,18] **Pc 4** can be taken up by membrane-mediated diffusion, and irradiated at 670 nm for PDT treatment. The triplet state energy transfer and generate singlet oxygen with occur with ~ 50% yield. [17]

In summary, a novel covalent NIR phototriggered Au NP-PDT drug delivery system has been designed for efficient delivery and controlled release. The **Pc 4** drug generation and release is based on the photolysis of the axial Si-C bond on the **Pc 227** molecule. Release is only possible into a lipophilic environment such as apoloar solvents or in cell membranes. No dark toxicity of the Au NP- **Pc 227** conjugates was observed in HeLa cell cultures. However, upon Vis-NIR irradiation, efficient PDT mediated cell death was observed. A detailed investigation of the relevant photophysics and photochemistry for this system is currently in progress. For in vivo application, the covalent attachment in a controlled manner is a favourable approach in drug delivery, which could minimize the non-specific drug accumulation and enhance the selectivity to the target. **Pc 227** , the “caged” **Pc 4** , is responsive to the near infrared light to release the active **Pc 4** . It can be covalently attached on the Au NPs via the Au-S bond and is physically more stable when compared to the **Pc 4** on the Au NPs. The in vivo efficacy of Au NP- **Pc 227** conjugates is currently under investigation. These results hold promise for the development of highly selective drug release through NIR induced photolytic bond cleavage.

Experimental Section

PEGylated Au NPs were synthesized through the modified Brust-Schiffrin method followed by ligand exchange with MeO-PEG-SH (MW5000). [18] The purified Au NPs were dissolved in chloroform and a 40 fold excess silicon phthalocyanine **Pc 227** (**Pc 227**) was added into the solution. After 48 hours mixing at room temperature, the solvent was removed under vacuum. The Au NP- **Pc 227** conjugates were suspended in aqueous solution and purified by centrifugation and 200 nm pore filters. The conjugates were characterized by UV-vis and fluorescence spectrometry. The average core size of Au NPs was identified by transmission electron microscopy. The singlet oxygen quantum yield of photolysis product of **Pc 227** was determined by decomposition of 1,3-diphenylisobenzofuran (DPBF) in methanol. [17] The irradiation wavelength for photolysis was 660 nm with a Clark MXR CPA laser to pump an optical parametric amplifier (OPA, Light Conversion). The **Pc 227** and photolysis samples were analysed by ESI-MS on a Thermo FINNIGAN LCQ DECA

with the ion transfer capillary temperature at 200 °C. [23,24] The m/z 200 to m/z 800 was monitored.

Cell experiments were carried out with a cervical epithelium cancer cell line originally derived from Henrietta Lacks (HeLa). Cells were cultured in the growth medium consisted of the following (final concentrations): Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. [25] Quantification of cell viability in the dark and after PDT was determined by MTT assays. 3×10^4 HeLa cells/well were seeded into two 96-well plates and incubated in the cell culture growth media for 24 hours. The **Pc 4**, **Pc 227** and Au NP-**Pc 227** conjugates in aqueous solution with the specified concentrations were added into the plates and incubated for another 24 hours. The following day, the cells were replaced with fresh growth media. One plate was kept in dark and the other was exposed to light (>600 nm) at 1 J cm^{-2} with the irradiation period of 12 min. The plates were returned for 24 hours in the incubator (37 °C, 5% CO_2). 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) reagent was then added into each well. After 4 hours incubation, the purple formazan crystals were formed by metabolic active cells. 100 μL of the solubilization solution (10% sodium dodecyl sulfate in 0.01 M HCl) per well was added to dissolve the cells and placed into the incubator overnight. The absorbance at 550 nm (the formazan salt) and 690 nm as the reference wavelength was measured by a microplate reader. 8 replicates were used for each condition. All experiments were repeated 3 times.

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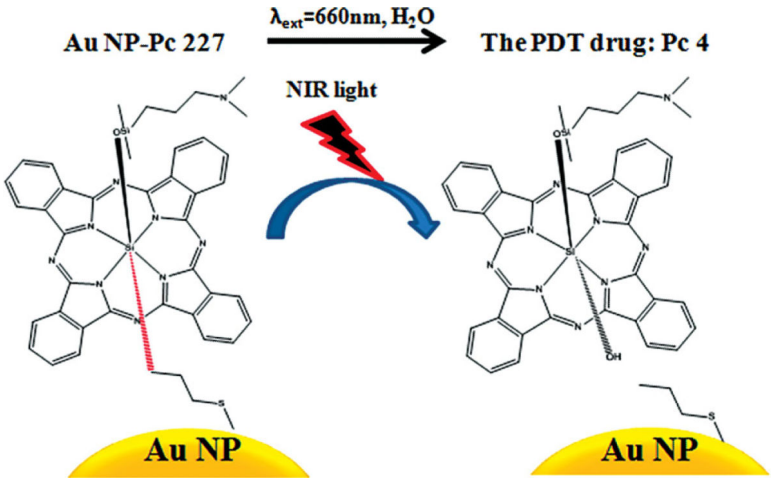

Scheme 1.

Schematic of phototriggered PDT drug **Pc 4** release from Au NP-**Pc 227** . The thiol group in **Pc 227** allows for covalent bonding of the drug precursor to the Au NP surface.

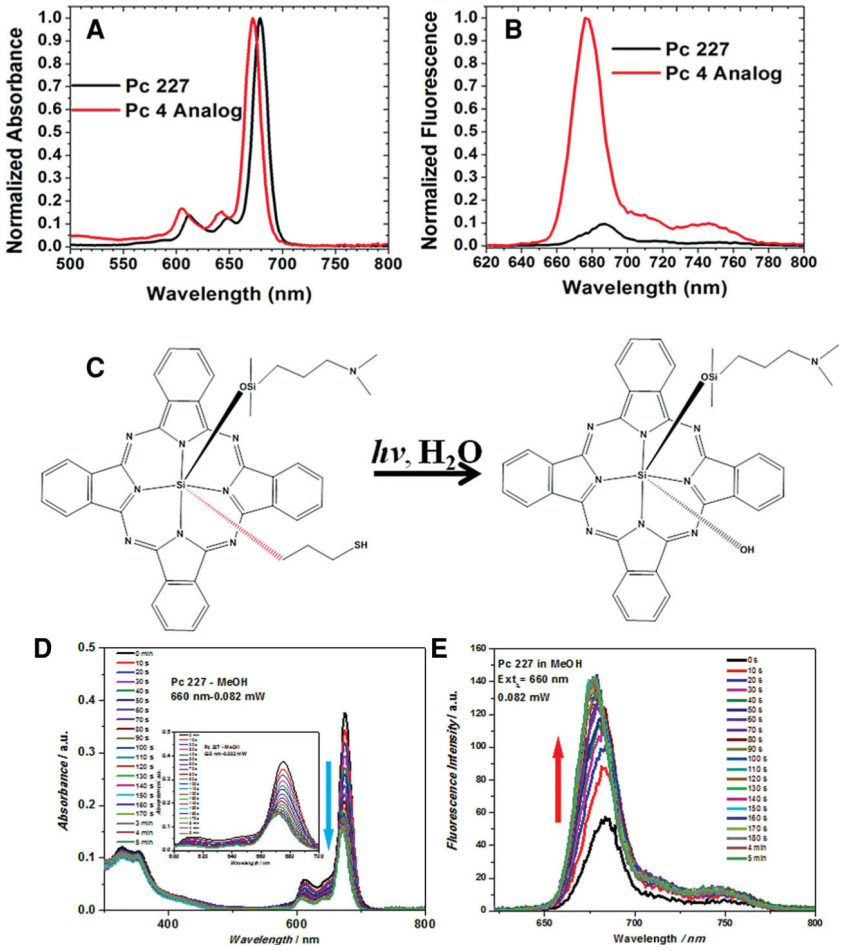

Figure 1.

Normalized absorbance (**A**) and fluorescence (**B**) spectra of **Pc 227** and the resulting photoproduct after irradiation with λ = 660 nm light (~3.5 mW cm $^{-2}$). (**C**) Schematic of homolytic bond cleavage and subsequent ligand exchange with water onto the central Si atomx to generate the PDT drug **Pc 4** , as confirmed by MS spectrometry. UV-Vis (**D**) and fluorescence (**E**) spectra of **Pc 227** in methanol before and after irradiation at 660 nm with the laser power of 0.082 mW.

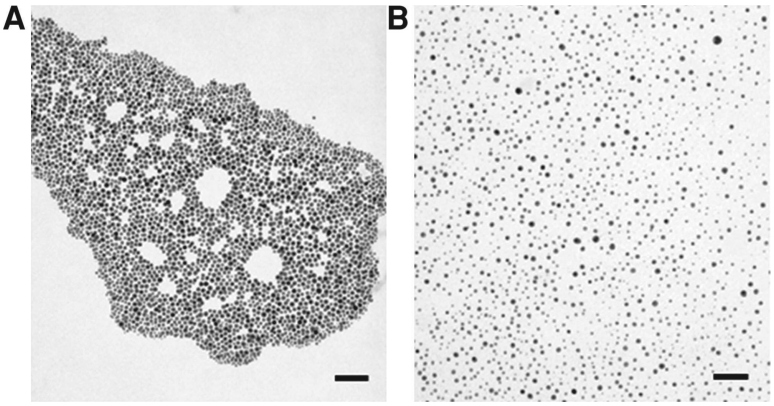

Figure 2.

TEM images of dodecylamine coated Au NPs (**A**) and Au **NP-Pc 227** conjugates (**B**). Scale bars: 50 nm.

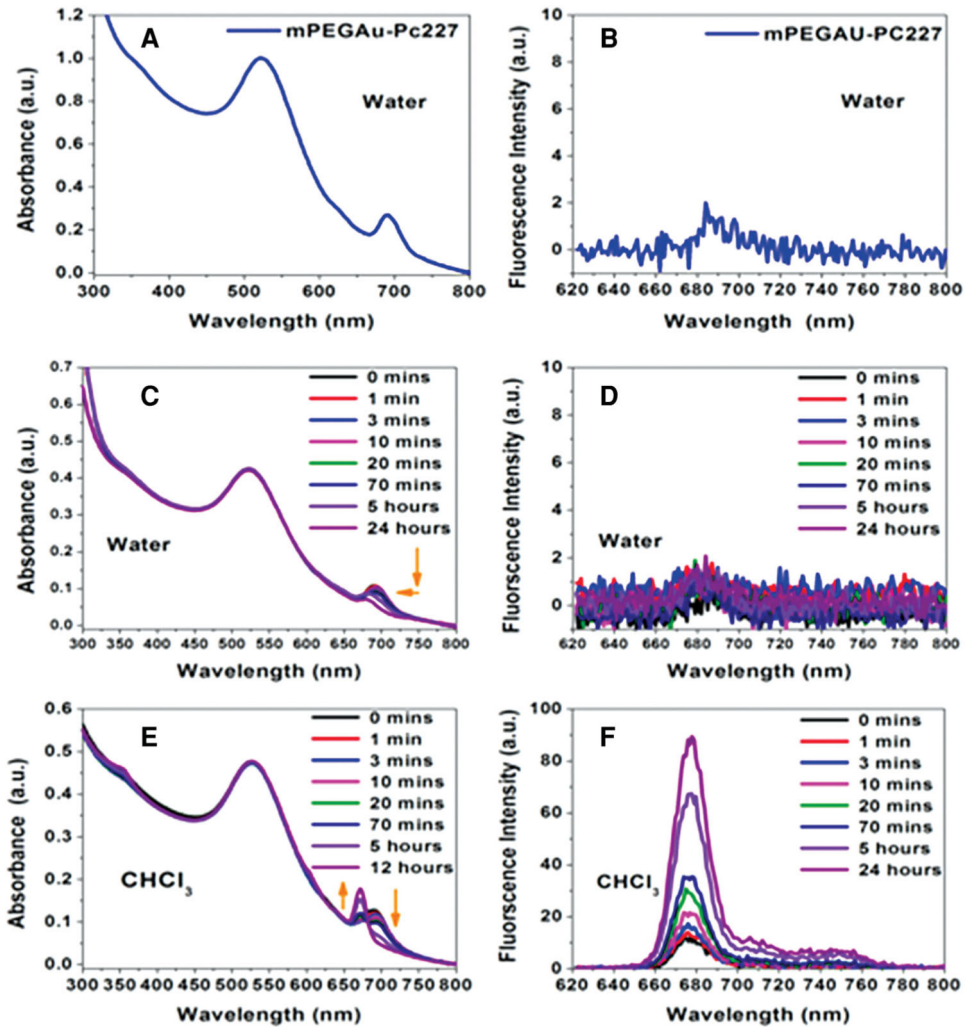

Figure 3.

Absorbance (**A**) and fluorescence (**B**) spectra of Au NP-Pc 227 conjugates in aqueous media, with overlapping spectra of both the NP (surface plasmon at 520 nm) and Pc 227 (690 nm absorbance) but with nearly complete quenching of fluorescence. NIR irradiation of Pc 227 in water (**C & D**) and chloroform (**E & F**) was determined through the new absorption band at ~671 nm and fluorescence at 680 nm.

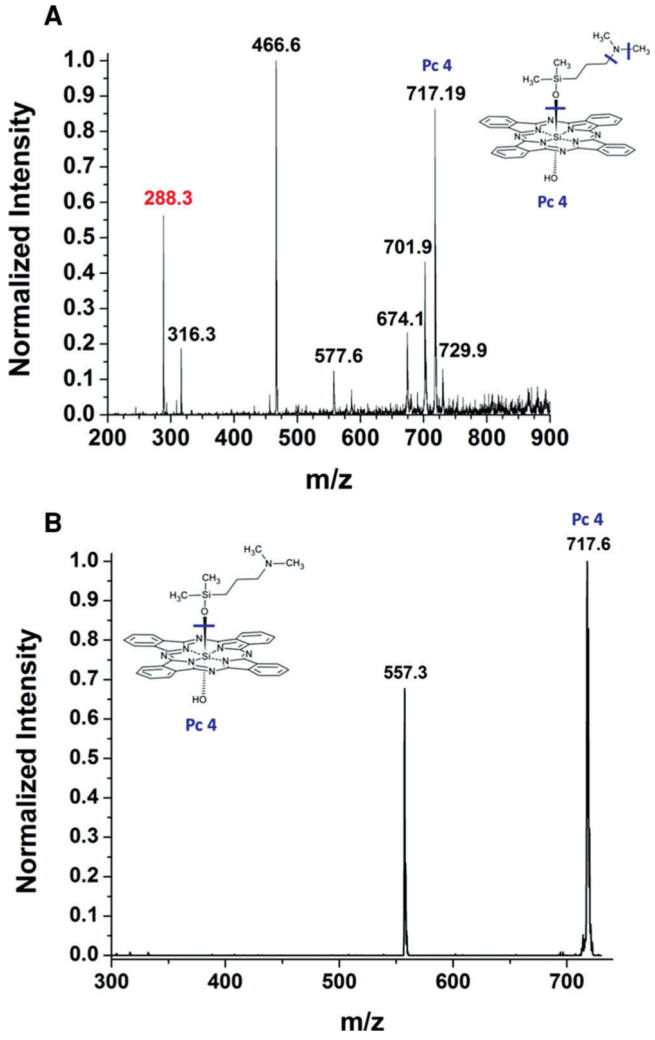

Figure 4.

ESI-MS Spectra of the Au NP-Pc 227 photoproduct after irradiation in water (**A**) and the corresponding m/z m/z ESI-MS Spectra of the Pc 227-Au NP photoproduct at 718 m/z (**B**).

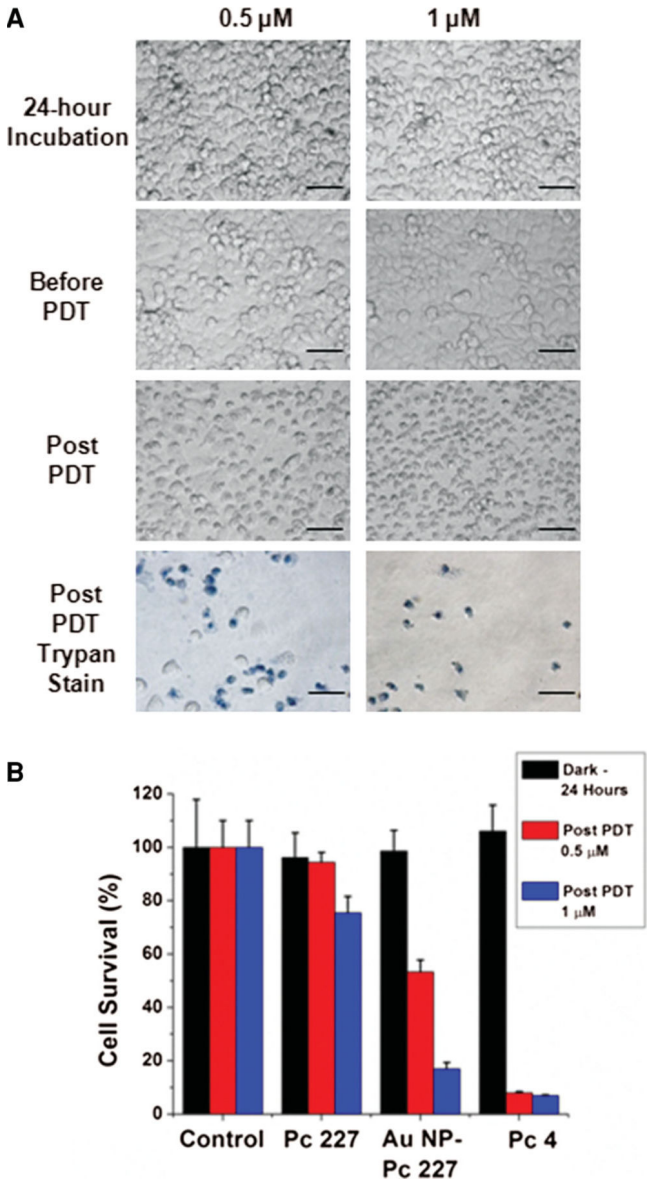

Figure 5.

Assessment of toxicity and PDT efficacy in HeLa cells via light microscopy, trypan blue staining (**A**), and MTT assays (**B**) after 1 μJ irradiation with > 600 nm light. Cells incubated with **Au NP - Pc 227** conjugates at 0.5 and 1 μM concentration after 24 hours remained healthy as indicated by cellular shape and trypan blue staining (A, top panel). Dramatic differences in cell morphology were observed by comparing optical images at 20X magnification before and after PDT treatment (**A**, middle panels).These results were further confirmed using trypan blue staining (**A**, bottom panel) and successfully quantified via MTT assays (**B**), with comparable efficacy to free **Pc 4** and improvement over free **Pc 227.** Scale bars: 100 μm.