

Versatile Photosensitizers for Photodynamic Therapy at Infrared Excitation

Peng Zhang,^{*,†} Wim Steelant,[‡] Manoj Kumar,[†] and Matthew Scholfield[†]

Laboratory of Nanomaterials Science and Laboratory of Biochemical & Biomedical Research, Department of Chemistry, New Mexico Tech, Socorro, New Mexico 87801

Received January 5, 2007; E-mail: pzhang@nmt.edu

Photodynamic therapy (PDT) is gaining acceptance as a technique for cancer treatment in recent years.^{1–3} PDT utilizes a photosensitizer, working as a light-sensitive drug, to treat the target tissue locally upon the irradiation of light with appropriate wavelengths. It is generally accepted that the mechanism of PDT is based on the interaction between the excited photosensitizer and surrounding molecules, generating reactive oxygen species (ROS), such as singlet oxygen (¹O₂). ROS can cause oxidative damage to biological substrates and ultimately cell death. One of the key components in effective and efficient PDT is the photosensitizers, also called PDT drugs. Features most desired for an ideal PDT drug include the following: (1) it must be specific to the target tissue; (2) have a high photochemical reactivity, that is, it can effectively produce ROS when exposed to appropriate irradiation; (3) exhibit little toxicity in the dark; and (4) can be excited at a wavelength in the region where tissue penetration of irradiation is at a maximum. Here we report the design of a type of versatile photosensitizers, which could potentially satisfy all of the above requirements and become the next generation PDT drugs, based on photon upconverting nanoparticles (PUNPs).

Photon upconverting materials convert lower-energy light to higher-energy light through excitation with multiple photons. They have been widely used for some time in such applications as display, bioassay, and bioimaging, to name a few.⁴ In this application, we take advantage of the fact that such materials would adsorb infrared irradiation and emit visible light to further excite the photosensitizing molecules. The particles of nanometer size would also facilitate the delivery and excretion of the proposed photosensitizer as a PDT drug.

The optimal spectral window for biological tissue penetration of irradiation is around 800 nm to 1 μm. Yet, single photons with infrared wavelengths are usually energetically too low for ¹O₂ generation. Multiphoton excitation using infrared light as irradiation source has been proposed and explored.^{5–9} Experiment to combine photon upconverting materials with a well-established PDT drug was mentioned, albeit with very limited efficiency in generating ¹O₂.¹⁰

The new design of versatile PDT photosensitizers we propose is schematically shown in Figure 1. PUNPs are first coated with a porous, thin layer of silica. During the coating process, photosensitizing molecules with high absorbance in the spectral window matching the emission of the PUNPs are doped, so that the resulting silica layer contains a certain amount of these photosensitizing molecules. Finally, an antibody, specific to antigens expressed on the target cell surface, is covalently attached to the silica-coated nanoparticles. When the thus-prepared nanoparticles are irradiated by infrared light, emission from the PUNPs will be absorbed by the photosensitizing molecules coated on their surfaces. Subsequently, excited photosensitizing molecules will interact with surrounding ground-state molecular oxygen, generating ¹O₂, leading to oxidative damage of the neighboring cells to which the nanoparticles are attached via specific antigen–antibody binding. As a proof of concept, we carried out experiments to demonstrate the feasibility of this design.

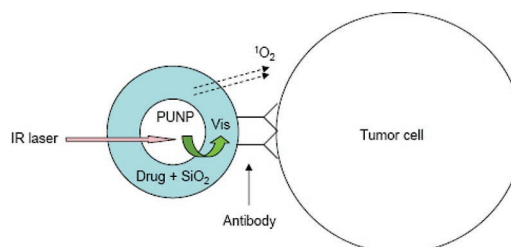


Figure 1. Schematic of the design of the versatile photosensitizer based on PUNPs. Dimensions not drawn to scale.

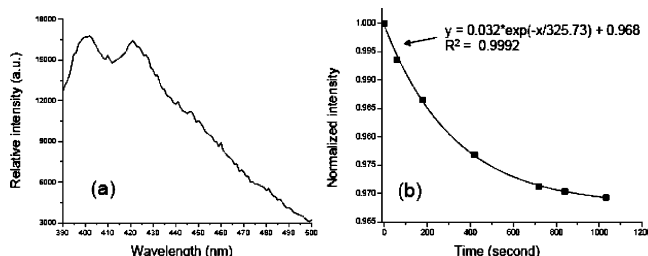


Figure 2. (a) Emission spectrum of ADPA, excited at 374 nm. (b) Change of normalized intensity of ADPA's emission peak at 402 nm over time when the mixture containing ADPA and M-540-coated PUNPs was irradiated by a 974 nm laser. The line represents the fitting of the data points, using the listed equation with correlation coefficient R^2 .

The PUNPs used in this study are NaYF₄:Yb³⁺,Er³⁺ nanoparticles, which have been recognized as one of the most efficient photon upconverting phosphors.¹¹ When excited by an infrared (974 nm) source, strong visible bands appear around 537 and 635 nm. The silica coating was realized through sol–gel reaction. Merocyanine 540 (M-540) is used as photosensitizing molecule, and doped into the silica layer during the coating process. M-540 can produce ¹O₂ and other reactive oxygen species and has been used before in PDT as a photosensitizer with a visible light source.¹² There is a good overlap between the NaYF₄:Yb³⁺,Er³⁺ nanoparticles' emission and M-540's absorption (see Supporting Information). The photon upconverting property of the nanoparticles was not affected by the silica coating, as confirmed by their photoluminescence spectrum. The presence of M-540 in the silica coating could be readily confirmed by the change in color of the nanoparticles, to slightly yellowish.

Generation of ¹O₂ by these PUNP-based photosensitizers was detected chemically, using the disodium salt of 9,10-anthracenedipropionic acid (ADPA) as a ¹O₂ sensor.¹³ When excited around 374 nm, the fluorescence emission of ADPA displays two strong bands centering around 400 and 420 nm. In the presence of ¹O₂, ADPA is bleached to its nonfluorescent endoperoxide. By measuring the decrease of the ADPA's fluorescence bands, we can monitor the generation of singlet oxygen.

Fluorescence measurements to detect the generation of ¹O₂ from the PUNP-based photosensitizers were carried out using a spectrofluorometer. An M-540-coated nanoparticles suspension in buffer

[†] Laboratory of Nanomaterials Science.

[‡] Laboratory of Biochemical & Biomedical Research.

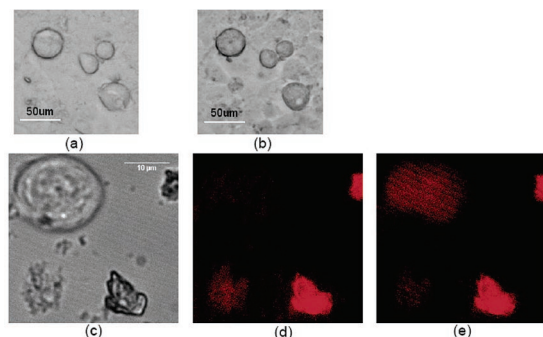


Figure 3. (a) MCF-7/AZ cells incubated with Trypan Blue and antibody-functionalized M-540-coated PUNPs modified with antibody on surface. (b) MCF-7/AZ cells in (a) after 974 nm laser irradiation for 36 min. (c) Optical image of MCF-7/AZ cells incubated with PI and M-540-coated PUNPs modified with antibody on surface. (d) Fluorescence image of MCF-7/AZ cells in (c) before 974 nm laser irradiation. (e) Fluorescence image of MCF-7/AZ cells in (c) after 974 nm laser irradiation for 43 min.

was mixed with a small amount of ADPA. The solution was first irradiated for several minutes at 540 nm using a xenon lamp, while the 402 nm emission peak of ADPA was measured at different time intervals. The decrease of the ADPA fluorescence intensity over time confirmed the $^1\text{O}_2$ generation from the M-540-coated nanoparticles under visible light irradiation matching M-540's absorption band. The solution was then illuminated by an infrared laser of 974 nm, which also led to the decrease of ADPA fluorescence intensity. The fluorescence emission of ADPA at 402 nm was measured at different time intervals, as shown in Figure 2.

It has been shown in previous reports that the intensity decrease of ADPA emission follows an exponential decay over time, as ADPA being quenched by the generated $^1\text{O}_2$.^{14,15} The data points in Figure 2 could be nicely fit into an exponential decay function. This suggests that the kinetics of the M-540-coated PUNPs generating $^1\text{O}_2$ is very similar to that of other silica-based nanoparticles described in the literature.^{14,15} The major difference is that the M-540-coated PUNPs were excited by IR irradiation, while the others were excited by visible irradiation. This difference would allow the penetration depth to increase several times for the PUNP-based PDT drugs, a highly desired feature in clinical applications.

In vitro PDT tests were performed to confirm the photodynamic cytotoxicity of the M-540-coated PUNPs toward MCF-7/AZ breast cancer cells. MCF-7/AZ cells express high levels of MUC1/episialin. A mouse monoclonal antibody, anti-MUC1/episialin, which is highly specific toward the MCF-7/AZ cells, was covalently attached to the M-540-coated PUNPs. Next, two sets of experiments were carried out. In the first experiment, MCF-7/AZ cells in suspension were incubated with Trypan Blue, a vitality dye that does not stain the cell unless the cell membrane is damaged, and the antibody-functionalized M-540-coated PUNPs. Subsequently, MCF-7/AZ cells were placed onto an inverted microscope and illuminated by an IR laser, coupled through the objective. Optical images of MCF-7/AZ cells were taken at different time intervals to monitor cell viability. Images of MCF-7/AZ cells before IR irradiation and after 36 min of IR irradiation are shown in Figure 3a and b, respectively. After IR irradiation, MCF-7/AZ cells displayed shrinkage and were stained with Trypan Blue, indicating induced cell death. In the second experiment, MCF-7/AZ cells in suspension were incubated with propidium iodide (PI), a fluorescence dye that stains the nuclei red once the cell is dead or dying, and the antibody-functionalized M-540-coated PUNPs. Fluorescence images of MCF-7/AZ cells were taken at different time intervals to monitor cell viability while they were illuminated by the IR laser. Images of MCF-7/AZ cells before IR irradiation and after 43 min of IR irradiation are shown

in Figure 3c–e (optical and fluorescence), respectively. The fluorescence of the cell in the upper left corner (Figure 3d) increased over time under IR irradiation, indicating the dying process of the cell. Note that, in both experiments, cells in the same dishes, undergoing same treatment with the antibody-functionalized M-540-coated PUNPs and respective dyes but without IR irradiation, do not display cell death during the same period of time, which confirms that the cell killing is directly associated with the IR irradiation. Control experiments with IR irradiation but without nanoparticles also showed that IR irradiation alone did not cause cell death.

These results demonstrate that photosensitizers of the proposed design have great potential to become the next generation PDT drugs. Compared to the PDT drugs currently available, the proposed PUNP-based photosensitizers display the following advantages: (1) Excitable at a wavelength in the infrared region where tissue penetration of light is several times deeper. (2) Highly specific. The photosensitizers are brought to the target through antigen–antibody interaction. (3) Expanding the pool of photosensitizing molecules as PDT drugs. The fact that photosensitizing molecules are encapsulated inside the silica layer allows the use of some molecules which would otherwise be unlikely candidates. In addition, there are a lot more green- or blue-absorbing than red-absorbing molecules available to be considered as PDT drugs. (4) Versatile for different types of tumors. There are a variety of antibodies and other ligands available for different types of tumors, which can be attached to the silica surface of the PUNPs to target the desired sites in vivo.¹⁶ Indeed, in vivo PDT studies using these newly designed photosensitizers are to be carried out next, along with the optimization of parameters, such as the type and amount of photosensitizing molecules loaded, the amount of antibody coated on the silica surface, and the intensity of the infrared irradiation.

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Supporting Information Available: Additional experimental details. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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