Highly Efficient, Conjugated-Polymer-Based Nano-Photosensitizers for Selectively Targeted Two-Photon Photodynamic Therapy and Imaging of Cancer Cells

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Abstract: Two-photon photodynamic therapy (2P-PDT) is a promising noninvasive treatment of cancers and other diseases with three-dimensional selectivity and deep penetration. However, clinical applications of 2P-PDT are limited by small two-photon absorption (TPA) cross sections of traditional photosensitizers. The development of folate receptor targeted nano-photosensitizers based on conjugated polymers is described. In these nano-photosensitizers, poly(9,9-bis[6-(bromohexyl)]fluorene-2,7-ylenevinylene)-co-alt-1,4-(2,5-dicyanophenylene), which is a conjugated polymer with a large TPA cross section, acts as a two-photon light-harvesting material to significantly enhance the two-photon properties of the doped photosensitizer tetraphenylporphyrin (TPP) through energy transfer. These nanoparticles displayed up to 1020-fold enhancement in two-photon excitation emission and about 870-fold enhancement in the two-photon-induced singlet oxygen generation capability of TPP. Surface-functionalized folic acid groups make these nanoparticles highly selective in targeting and killing KB cancer cells over NIH/3T3 normal cells. The 2P-PDT activity of these nanoparticles was significantly improved, potentially up to about 1000 times, as implied by the enhancement factors of two-photon excitation emission and singlet oxygen generation. These nanoparticles could act as novel two-photon nano-photosensitizers with combined advantages of low dark cytotoxicity, targeted 2P-PDT with high selectivity, and simultaneous two-photon fluorescence imaging capability; these are all required for ideal two-photon photosensitizers.

Introduction

Photodynamic therapy (PDT) is a promising noninvasive treatment of cancers and other diseases.[1] In PDT, drug molecules (photosensitizers) are selectively activated by a localized light beam. The activated photosensitizers undergo a series of physophysical processes, and subsequently, transfer the excitation energy to molecular oxygen, which generates reactive oxygen species, such as singlet oxygen, to kill nearby cells. The photosensitizers could be activated by one- (1PE) or two-photon excitation (2PE) processes. The two-photon excitation photodynamic therapy (2P-PDT) techniques are advantageous over the traditional one-photon counterparts because they offer deeper penetration into body tissues, more confined treatment areas, and three-dimensional spatial selectivity to reduce adverse effects to nearby normal tissues.[2] However, the clinical applications of 2P-PDT are hindered by the limited two-photon-induced singlet oxygen generation capability of current photosensitizers due to their small two-photon absorption (TPA) cross sections.[3] Much effort has been devoted to the development of novel two-photon photosensitizers with large TPA cross sections.[4] Alternatively, the 2P-PDT activity of conventional photosensitizers can be enhanced through 2PE fluorescence resonance energy transfer (2PE-FRET) processes by developing various nano-photosensitizers, such as dendrimers,[5] organically modified silica nanoparticles (NPs),[6] quantum dots,[7] and copolymer micelles.[8] The development of photosensitizers with a high two-photon singlet oxygen generation capability is still of great scientific interest and practical importance to ultimately realize the clinical applications of 2P-PDT. Nano-photosensitizers are attractive because of their potential multifunctional capability, which results in the integration of efficient nano-photosensitizers with specific targeting and 2PE fluorescence imaging capabilities to allow imaging-guided PDT with high selectivity.[9]

Conjugated polymers have found wide applications due to their large extinction coefficients and high fluorescence quantum yields.[10] In particular, conjugated polymers display optical amplification through FRET processes.[11] This feature has been widely utilized to develop various biosensing and bioimaging schemes with enhanced detection efficiency.[12] Dye-doped conjugated polymer NPs have attracted much attention due to their unique intraparticle energy-transfer processes,[12] in which energy harvested by the conjugated polymer can be three-dimensionally transferred to the doped acceptor molecules inside the NP. Conjugated polymers also have much larger TPA cross sections than their small-molecule counterparts[13,14] and can act as two-photon light-harvesting materials to enhance
two-photon emission of the nearby acceptor molecules by up to hundreds of times through 2PE-FRET processes; this has been utilized for various two-photon applications, such as two-photon sensing, imaging, and PDT, with enhanced efficiency[9b,c,14]. Photosensitizer-doped conjugated polymer NPs have been demonstrated to act as novel two-photon photosensitizers with simultaneous 2PE fluorescence imaging capability[9c]. So far, the two-photon-induced singlet oxygen generation performances of these NPs still have much room for improvement, considering the moderate TPA cross-section values of current conjugated polymers. We recently reported a novel cationic conjugated polymer, poly[9,9-bis(6’-(6,12-dicyanophenylene)-2,5-dicyanophenylene)co-alt-1,4-(2,5-dicyanophenylene)], which displayed a TPA cross section of about 891 GM per repeat unit at $\lambda = 810$ nm and could generate singlet oxygen at the same time.[15] Although this conjugated polymer could be utilized for 2P-PDT, its intrinsic singlet oxygen generation efficiency (≈6.9%) was significantly lower than that of typical clinical photosensitizers (generally 30–80%).[14] Furthermore, most of the reported two-photon photosensitizers lack specific targeting to cancer cells because uptake is mainly through nonspecific interactions with cells.

Herein, we developed highly efficient two-photon nano-photosensitizers by encapsulating tetraphenylporphyrin (TPP) into NPs by using a hydrophobic conjugated polymer, poly[9,9-bis(6’-(bromohexyl)-2,7-ylenylene)-co-alt-1,4-(2,5-dicyanophenylene)] (PFVCN), as the matrix. This hydrophobic polymer has the same backbone as the previously reported cationic conjugated polymer that displayed a large TPA cross section.[15] This approach combines the advantages of conjugated polymers (large TPA cross section, but low singlet oxygen generation efficiency) and traditional photosensitizers (high singlet oxygen generation efficiency, but small TPA cross section) to achieve an even higher two-photon-induced singlet oxygen generation capability for efficient 2P-PDT. In these nano-photosensitizers, efficient intraparticle energy transfer from PFVCN to TPP helps to enhance two-photon-induced singlet oxygen generation of the doped TPP. The drawback of the conjugated polymers and photosensitizers alone were overcome by 2PE-FRET processes. The singlet oxygen generation efficiency of these NPs was evaluated by a chemical method as well as directly monitoring the characteristic emission of singlet oxygen at $\lambda = 1270$ nm. Two-photon-induced singlet oxygen generation of TPP in these NPs was enhanced by about 870-fold by using PFVCN as the two-photon light-harvesting material. The effective TPA cross-section of PFVCN in the NPs was smaller than that in solution due to distortion of the conjugated structures inside the NPs. The emission spectrum of PFVCN in the NPs was smaller than that in solution due to distortion of the conjugated structures inside the NPs. The emission spectrum of PFVCN has a reasonable overlap with the absorption spectrum of TPP (Figure 1 b), which allows energy transfer from two-photon-excited PFVCN to TPP to enhance the two-photon optical properties of TPP. PSMA results in negatively charged carboxyl groups on the NP surface to stabilize the NPs. DSPE-PEG2000-FA was incorporated onto the surface of the NPs to allow specific targeting of cancer cells and to improve the biocompatibility of the NPs.[16] Dynamic light

Results and Discussion

The procedure for the preparation of photosensitizer-doped conjugated polymer NPs is illustrated in Figure 1 a and described in detail in the Supporting Information. In these NPs,

![Figure 1](https://example.com/figure1.jpg)

(a) Schematic illustration of the preparation of photosensitizer-doped conjugated polymer NPs for selectively targeted 2P-PDT. PSMA = poly(styrene-co-maleic anhydride), DSPE-PEG2000-FA = 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)-2000]. b) Absorption and emission spectra of PFVCN and TPP. c) Size distribution (mean number percentage) of FA-PFVCN/TPP NPs.
scattering (DLS) measurements indicated that the average size of the NPs were about 28 nm (Figure 1 c), which was consistent with results obtained by TEM measurements (average size of \( \approx 27 \) nm, see Figure S2 in the Supporting Information).

PFVCN/TPP NPs with different TPP doping concentrations (molar ratio of 0–4% with respect to the repeat unit of PFVCN) were prepared to investigate the FRET process from PFVCN to TPP inside the NPs. Under excitation at the absorption maximum of PFVCN, \( \lambda = 469 \) nm, the emission of PFVCN (\( \lambda = 500–620 \) nm) decreased steadily as the TPP concentration increased; this was accompanied by the appearance of a new emission band in the \( \lambda = 640–720 \) nm range. Because TPP molecules themselves have little absorption at \( \lambda = 469 \) nm and cannot be directly excited, the \( \lambda = 640–720 \) nm emission band mainly arises from energy transfer from PFVCN to TPP (Figure 2 a). The energy-transfer efficiency (ET) can be estimated based on quenching of the donor (PFVCN) emission, according to \( \text{ET} \% = \frac{1 - F/F_0}{1} \times 100 \% \), in which \( F_0 \) and \( F \) are the emission intensities of PFVCN in the absence and presence of TPP, respectively. The intra-NP energy transfer from PFVCN to TPP, which was facilitated by the three-dimensional nature of the NPs, displayed high efficiency despite relatively little spectral overlap between the PFVCN emission and TPP absorption. The energy-transfer efficiency increased with increasing TPP molar ratio and reached 93% in the presence of 4% TPP. As the molar ratio of TPP increased, the TPP emission intensity in PFVCN/TPP NPs steadily increased until saturation was reached when the TPP molar ratio was higher than 2% due to the self-quenching effect (Figure S3 in the Supporting Information). An enhancement factor can be defined as the ratio of the TPP emission intensity in PFVCN/TPP NPs (\( \lambda_{2PE} = 469 \) nm) to that in TPP NPs containing the same amounts of TPP (using PSMA and DSPE-PEG2000 as the matrix and surfactant, respectively) under excitation at the absorption maximum of TPP (\( \lambda = 416 \) nm). The enhancement factor reached up to 4.2 when the TPP molar ratio was 0.25% (Figure 2 c).

The effects of using PFVCN as the two-photon light-harvesting material to enhance the two-photon properties of TPP were investigated by measuring the 2PE fluorescence spectra of these PFVCN/TPP NPs by using femtosecond laser pulses at \( \lambda = 810 \) nm as the excitation source. The 2PE emission spectra of PFVCN/TPP NPs (Figure 2 b and Figure S4 in the Supporting Information) displayed a similar trend to that of the 1PE emission spectra: as the molar ratio of TPP increased, the emission intensity of PFVCN in PFVCN/TPP NPs steadily decreased, and was accompanied by the appearance of sharp emission bands between \( \lambda = 640 \) and 720 nm (Figure 2 b). The observed \( \lambda = 640–720 \) nm emission band is mainly due to 2PE-FRET from PFVCN to TPP because the 2PE fluorescence of the same amount of TPP NPs without PFVCN is much weaker due to the small TPA cross section of TPP. The 2PE emission from TPP in PFVCN/TPP NPs was significantly enhanced by FRET with PFVCN as the 2PE light-harvesting material. The two-photon emission enhancement factor was estimated to be as high as 1020 for PFVCN/TPP NPs with a TPP molar ratio of 0.25%. The TPA cross-section value for bare TPP molecules is reported to be about 12 GM,\[29] the effective TPA cross section is thus estimated to be about 12000 GM per TPP molecule when assuming no change in the emission yield of TPP molecules. The large enhancement in the 2PE emission of TPP can be ascribed to the much larger TPA cross section of PFVCN than that of TPP and multiple PFVCN repeat units acting as light-harvesting materials for one TPP molecule.

Porphyrin-type molecules, such as TPP, can be utilized to act as effective photosensitizers for PDT.\[3] The application of porphyrin-type photosensitizers in 2P-PDT is limited by their small TPA cross sections. The observed significantly enhanced 2PE emission of TPP molecules inside these PFVCN/TPP NPs sug-
gests that these NPs are appealing nano-photosensitizers for 2P-PDT. An essential prerequisite for efficient nano-photosensitizers is a high singlet oxygen generation capability. The 1PE singlet oxygen generation capability of these PFVCN/TPP NPs was evaluated by monitoring the characteristic emission of singlet oxygen at $\lambda = 1270$ nm in D$_2$O. Under excitation at the absorption maximum of PFVCN ($\lambda = 469$ nm), PFVCN/TPP NPs showed a strong emission at $\lambda = 1270$ nm, which indicated efficient singlet oxygen generation. The emission intensity of singlet oxygen generated by PFVCN/TPP (0.25 %) NPs at $\lambda = 469$ nm was enhanced by a factor of 4.5 relative to that of TPP NPs with same amount of TPP under excitation at its absorption maximum of $\lambda = 416$ nm (Figure 3 a). Cationic PFVCN molecules were previously reported to display singlet oxygen generation capability in aqueous solution.\textsuperscript{[15]} We have tested the singlet oxygen generation capability of PFVCN NPs (without doped TPP) under excitation at $\lambda = 469$ nm and observed no emission at $\lambda = 469$ nm (Figure 3 a), which suggests very little singlet oxygen generation directly from PFVCN inside the NPs. The reduced singlet oxygen generation by PFVCN NPs might be due to the aggregation effect of conjugated polymers inside the NPs. The energy-transfer process between the aggregated polymer chains inside the NPs competes with the intersystem crossing process, which reduces the yield of PFVCN triplet excited states that are essential for singlet oxygen generation. The enhancement in singlet oxygen generation is consistent with the enhancement factors of TPP emission for PFVCN/TPP for different molar ratios of TPP under 1PE (Figure 3 b and Figure S5 in the Supporting Information). This result confirms that energy transfer from PFVCN to TPP helps to enhance the excitation efficiency of TPP, which subsequently undergoes a series of photophysical processes to generate singlet oxygen.

By considering the fact that the 2PE emission of TPP in PFVCN/TPP NPs was enhanced by over 1000 times through FRET (Figure 2 b), PFVCN/TPP NPs are expected to display a similar extent of enhancement in singlet oxygen generation under 2PE. The two-photon-induced singlet oxygen generation capability of PFVCN/TPP was evaluated by using a chemical method to monitor the photo-oxidation of 9,10-anthracenediybis(methylene)dimalonic acid (ABDA) in the presence of photosensitizers. ABDA converts into its endoperoxide in the presence of $^{1\text{O}}_2$, which leads to a decrease in ABDA absorption.\textsuperscript{[17]} Under illumination of femtosecond laser pulses at $\lambda = 810$ nm in the presence of PFVCN/TPP (0.25 %) NPs, ABDA degraded rapidly. More than 80 % of ABDA was photo-oxidized after irradiating the sample for 90 min, which indicated efficient two-photon-induced singlet oxygen generation. In contrast, ABDA in the presence of PFVCN NPs (without TPP) or TPP NPs (without PFVCN) under the same irradiation conditions showed little decrease after femtosecond laser irradiation for up to 120 min (Figure 4); this indicates that PFVCN or TPP alone has a much lower two-photon-induced singlet oxygen generation capability. The photo-oxidation rate of ABDA in the presence of PFVCN/TPP (0.25 %) NPs, PFVCN NPs, and TPP (2 %) NPs; $\lambda_{\text{ex}} = 810$ nm.

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FA is known to display a high binding affinity to folate receptors on the cell surface of many human tumors. The folate receptor is a tumor marker that is highly overexpressed in malignant tissues of epithelial origin relative to normal tissues. FA has emerged as an effective targeting ligand for the selective delivery of imaging and therapeutic agents to cancer tissues. The folic acceptor mediated uptake of NPs has been well studied. A small fraction of DSPE-PEG-FA (0.03–0.5%) is optimal for the effective delivery of NPs into folate receptor positive cancer cells. DSPE-PEG2000-FA was thus incorporated onto the surface of the PFVCN/TPP NPs (labelled FA-PFVCN/TPP NPs) to improve its specific targeting to cancer cells. We used an optimized formula to demonstrate specific targeting of cancer cells. The optimum amount of FA molecules per NP was roughly estimated to be 160–320 based on the NP size and the amount of PFVCN and DSPE-PEG2000-FA.

The incorporation of surface folate significantly facilitated the uptake of NPs in KB cancer cells (containing overexpressed folate receptors) and normal NIH/3T3 cells, which were monitored by confocal fluorescence imaging and 2PE fluorescence imaging. After incubation of FA-PFVCN/TPP (0.25%) NPs (0.5 µm in terms of PFVCN repeat units) with KB cancer cells and NIH/3T3 cells for 6 h, the cell nuclei were stained with Hoechst 33342, which is a popular cell-permeant nuclei counterstain that emits blue fluorescence when bound to double-stranded DNA. Confocal fluorescence cell imaging was conducted under excitation of Hoechst 33342 at λ = 405 nm, and emission signals from λ = 420 to 470 nm (from Hoechst 33342 only) were detected. The 2PE fluorescence imaging was conducted under excitation at λ = 860 nm to excite PFVCN only, and emission signals from λ = 500 to 550 nm were detected to avoid contributions from Hoechst 33342. The 2PE fluorescence imaging can thus be utilized to monitor the uptake of NPs. Strong 2PE fluorescence signals were observed in KB cancer cells, which indicated that FA-PFVCN/TPP (0.25%) NPs were effectively taken up by the KB cancer cells (Figure 5a, middle). In contrast, almost no 2PE fluorescence signal was observed from normal NIH/3T3 cells (Figure 5b, middle); this indicated that very few FA-PFVCN/TPP (0.25%) NPs were taken up by normal NIH/3T3 cells. Control experiments have been done on KB cancer cells incubated with PFVCN/TPP (0.25%) NPs without surface folate under the same conditions (Figure 5c), and no 2PE fluorescence signals were observed. This result confirmed that the incorporation of surface folate significantly facilitated the uptake of NPs by folate receptors overexpressed in KB cancer cells, and enabled specific targeting of cancer cells.

The two-photon photodynamic therapy (2P-PDT) activities of various NPs were evaluated by the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay (Figure 6). The FA-PFVCN/TPP (0.25%) NPs did not show any dark cytotoxicity at a concentration of up to 2 µm (in PFVCN repeat units, see Figure S6 in the Supporting Information).

Under illumination of femtosecond laser pulses at λ = 810 nm, the viability of KB cells incubated with 1.0 µm FA-PFVCN/TPP (0.25%) NPs rapidly decreased as the irradiation time increased. The cell viability decreased to about 42% under femtosecond laser irradiation for 15 min. In contrast, the viability of KB cells incubated with FA-TPP (0.25%) NPs (without folate) and FA-TPP NPs. The 2P-PDT activity was evaluated by using femtosecond laser pulses at λ = 810 nm.
significantly enhanced the two-photon-induced singlet oxygen generation capability, and thus, dramatically improved the 2P-PDT activity for cancer cells. This result is consistent with the fact that the two-photon-induced singlet oxygen generation capability of PFVCN/TPP (0.25%) is much higher than that of TPP (0.25%) NPs (without PFVCN; Figure 4). Control experiments were performed on normal cells (NIH/3T3) incubated with FA-PFVCN/TPP (0.25%) NPs and KB cancer cells incubated with PFVCN/TPP (0.25%) NPs (without folate). In both control experiments, the cell viability remained at nearly 100% and showed little 2P-PDT activity after irradiation for 15 min. These results are consistent with 2PE fluorescence cell imaging results (Figure 5b and c) that these two types of NPs are not effectively taken up by the cells.

Conclusion

We developed one type of photosensitizer-doped conjugated polymer NPs with a significantly enhanced two-photon-induced single oxygen generation capability to act as efficient two-photon nano-photosensitizers for targeted 2P-PDT and 2PE fluorescence imaging. In these NPs, PFVCN, which is a conjugated polymer with a large TPA cross section, acts as a two-photon light-harvesting material to significantly enhance the two-photon properties of the doped photosensitizer TPP through 2PE FRET processes. These NPs displayed up to 1020-fold enhancement in 2PE emission and about 870-fold enhancement in 2PE fluorescence imaging. These NPs were significantly improved relative to that without the use of conjugated polymers. The improvement in 2P-PDT efficiency could be potentially up to about 1000 times, as implied by the enhancement factors of 2PE emission and singlet oxygen generation. These NPs could act as novel two-photon nano-photosensitizers with combined advantages of low dark cytotoxicity, selectively targeted 2P-PDT, and simultaneous 2PE fluorescence imaging capability; these are required for ideal two-photon photosensitizers. Herein, we demonstrated excellent performance of these nano-photosensizers on the cellular level. Considering the unique advantages of 2PE in applications for in vivo imaging and treatment, these photosensitizer-doped conjugated polymer NPs hold great potential for high spatial resolution imaging guided 2P-PDT with deep penetration for practical clinical applications.

Experimental Section

Materials

TPP, PSMA, cumene-terminated PSMA (average $M_n \approx 1900$), ABDA, deuterium oxide, and THF were purchased from Sigma Aldrich. DSPE-PEG2000-FA and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000) were purchased from Avanti Polar Lipids. Dulbecco’s modified eagle medium (DMEM), fetal bovine serum, streptomycin, penicillin, phosphate buffered saline (PBS), and FA-free RPMI-1640 medium were purchased from Invitrogen. PFVCN was synthesized as described in the literature.

Preparation of TPP-doped conjugated polymer NPs

The conjugated polymer NPs were prepared by using a modified re-precipitation method. To prepare TPP-doped conjugated polymer NPs (PFVCN/TPP NPs), different amounts of a solution of TPP in THF (0.1 mM) were mixed with a stock solution of PFVCN in THF (2.0 mM; 40 μm in repeat units) and a solution of PSMA (8.0 μL, 0.2 mM) and DSPE-PEG2000 (4 μL, 1.0 mg mL$^{-1}$) in THF. The mixtures were quickly added to deionized water (8 mL) under sonication and then left for 4 h before removal of THF by vacuum evaporation. Folate-functionalized NPs (FA-PFVCN/TPP NPs) were prepared by replacing DSPE-PEG2000 with DSPE-PEG2000-FA. The NP solutions were a clear, light-yellow color and remained stable for more than two weeks.

Characterization

UV/Vis absorption and fluorescence spectra were measured by using a Shimadzu UV/Vis spectrophotometer and a Jobin-Yvon Fluoromax-4 spectrofluorometer, respectively. The size distribution of the NPs was measured by DLS (Zetasizer Nano ZS, Malvern).

2PE fluorescence measurements

The 2PE fluorescence measurements were performed by using an Avesta TIF-100M femtosecond Ti:sapphire oscillator as the excitation source. The output laser pulses had a pulse duration of about 80 fs and a repetition rate of 84.5 MHz in the wavelength range from $\lambda = 750$ to 850 nm. The laser beam was focused onto the sample contained in a cuvette with a path length of 1.0 cm. The emission was collected at an angle of 90° to the incoming excitation beam by a pair of lenses and an optical fiber connected to a monochromator (Acton, Spectra Pro 2300i) charge-coupled device (Princeton Instruments, Pixis 100B) system. A short-pass filter with a cutoff wavelength at $\lambda = 750$ nm was placed before the spectrometer to minimize scattering from the pump beam. Fluorescein in water (pH 11), which was well characterized in the literature, was used as a reference (r). The TPA cross section ($\delta$) of the sample was calculated at each wavelength according to Equation (1), in which $S$ is the integrated 2PE fluorescence intensity, $\phi$ is the fluorescence quantum yield, and $C$ is the concentration of the sample (s) and reference (r). The concentration of the solution was in the range of 1.0 to 2.0 μM. The uncertainty in the measurement of the cross sections was about 15%.

Detection of singlet oxygen

The 1PE singlet oxygen generation was directly monitored by the characteristic emission of singlet oxygen at $\lambda = 1270$ nm in D$_2$O. The singlet oxygen emission was measured by using a Fluorolog-3 iHR spectrofluorometer (Jobin-Yvon) equipped with a near-infrared sensitive photomultiplier (Hamamatsu model: R5509-72) operated at $-80$ °C. A long-pass filter with a cutoff wavelength at $\lambda = 850$ nm was placed before the detector.

Two-photon-induced singlet oxygen generation was monitored by the chemical oxidation of ABDA in the aqueous dispersions of NPs. The decrease in the ABDA absorbance was monitored under irradiation with the 2PE device (Princeton Instruments, Pixis 100B) system. A short-pass filter with a cutoff wavelength at $\lambda = 750$ nm was placed before the spectrometer to minimize scattering from the pump beam. Fluorescein in water (pH 11), which was well characterized in the literature, was used as a reference (r). The TPA cross section ($\delta$) of the sample was calculated at each wavelength according to Equation (1), in which $S$ is the integrated 2PE fluorescence intensity, $\phi$ is the fluorescence quantum yield, and $C$ is the concentration of the sample (s) and reference (r). The concentration of the solution was in the range of 1.0 to 2.0 μM. The uncertainty in the measurement of the cross sections was about 15%.

$$\delta = \frac{S_s}{S_r} \times \frac{\phi_r}{\phi_s} \times \frac{C_r}{C_s} \times \delta_s$$
ation with femtosecond laser pulses at $\lambda = 810$ nm. The sample solution was prepared by combining the NP dispersions (1.0 mL) in water with a stock solution of ABDA (0.1 mL) in water (0.5 mM). The laser beam was focused onto a cuvette (1 cm path length) containing the solution (0.5 mL).

Cell culture

KB cancer cells (a gift from Dr. Lanny Yung Lin Yue, Department of Chemical and Biomolecular Engineering, NUS) were cultured in FA-free RPMI-1640 medium supplemented with fetal bovine serum (10%), streptomycin (100.0 mg L$^{-1}$), and penicillin (100 IU mL$^{-1}$). Normal NIH/3T3 fibroblast cells were cultured in DMEM supplemented with fetal bovine serum (10%), streptomycin (100.0 mg L$^{-1}$), and penicillin (100 IU mL$^{-1}$). Cells were maintained in a humidified atmosphere of 5% CO$_2$ at 37°C.

Proliferation assay

Cell viability was determined by using the XTT colorimetric cell proliferation kit (Roche) following the manufacturer’s guidelines. Briefly, cells were grown to 20–30% confluence (they would reach 90% confluence within 24 h in the absence of compounds) in 96-well plates. The medium was aspirated and then treated with media (0.1 mL) containing different amounts of NPs. After incubation with femtosecond laser pulses at $\lambda = 210$ nm, the sample solution was prepared by combining the NP dispersions (1.0 mL) in water with a stock solution of ABDA (0.1 mL) in water (0.5 mM). The laser beam was focused onto a cuvette (1 cm path length) containing the solution (0.5 mL).

KB cancer cells or normal NIH/3T3 cells were separately seeded on glass-bottomed dishes (Mattek) and grown until 70–80% confluence. The NPs (0.5 mL) were further incubated for 24 h followed by the XTT assay of cell proliferation. Cell experiments without NPs under the same experimental conditions were performed. Cell experiments without NPs under the same experimental conditions were performed. Cell experiments without NPs under the same experimental conditions were performed.

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