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Dual Wavelength Stimulation of Polymeric Nanoparticles for Photothermal Therapy

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Abstract

Background and Objective—Photothermal therapy (PTT) has several applications in the areas of wound healing, pain management, bacterial infection control, and cancer treatment dependent on the temperature that is generated. PTT is often used exclusively with near infrared (NIR) light and most nanoparticles (NP) used for PTT are designed to absorb within one narrow range of wavelengths. We have developed a dual-wavelength photo-thermal therapy by capitalizing on the dual absorption of nanoparticles in the blue and NIR range.

Materials and Methods—Our lab has previously developed NP based on the semiconducting, conjugated polymer poly-[4,4-bis(2-ethylhexyl)-cyclopenta[2,1-b;3,4-b']dithiophene-2,6-diyl-alt-2,1,3-benzoselenadiazole-4,7-diyl] (PCPDTBSe). The NP have strong absorption in the blue and NIR regions. In this report, we have explored the heat generated by PCPDTBSe NP using simultaneous delivery of 450 and 800nm light, either independently or together for photothermal ablation of mouse colorectal cancer cells.

Results—The heat generation studies indicated that the use of either 450 or 800 nm wavelengths at the same fluences produced approximately the same temperature change of deionized water. Fluences of 114.6 and 229.2 J/cm², utilizing 450 or 800 nm light applied individually resulted in temperatures of 8–47°C above ambient temperature, leading to a 90% reduction in cell viability. Simultaneous stimulation of the PCPDTBSe NP with 450 and 800 nm light effectively doubles the effective power delivered, resulting in temperatures 18–63°C above ambient and 100% photothermal ablation of the colorectal cancer cells.

Conclusion—The results of this study demonstrate that PCPDTBSe polymer NP can be utilized as effective PTT agents by capitalizing on their dual absorption of both blue and NIR light. *Lasers Surg. Med.* 48:893–902, 2016.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Keywords

blue light; colorectal cancer; dual wavelength; nanoparticles; near infrared light; photothermal therapy

INTRODUCTION

Photothermal therapy (PTT) is a rapidly emerging field for cancer treatment *via* the application of mild and ablative hyperthermia. PTT using nanoparticles (NP) that absorb near infrared (NIR) light and generate heat has been extensively studied in the literature, and temperature increases are dependent on the concentration of photothermal agent, laser power density, and treatment time. PTT utilizing lower temperatures (up to 45°C) is classified as mild-hyperthermia has been shown to be effective for wound healing and pain management. On the other hand, ablative temperatures (onset of irreversible protein denaturation begins at 45°C) are beneficial for treating diseases such as cancer (Photothermal ablation (PTA)) [1–3]. Existing PTT agents can be divided into subgroups of organic molecules, including conjugated polymers such as Poly(3,4-ethylenedioxythiophene), polyaniline, and polypyrrole; [4–7] NIR absorbing dyes such as Prussian blue and [8] Indocyanine green; [9] gold and silver based nanoparticles, nanorods and nano-cages; [10,11] and carbon based nanomaterials such as carbon nanotubes [12–14] and graphene [15].

One of the advantages of nanoparticles for delivery of PTT is that their small size can facilitate their potential to passively localize in tumors. It has been proposed in the literature that this may be due to the enhanced permeability and retention (EPR) effect, in which small particles can extravasate from the leaky vasculature of tumors to accumulate and remain trapped within the tumor due to vessel tortuosity [16,17]. Since some nano-particles can effectively localize in tumor tissue passively *via* the EPR effect, they do not necessarily need to be uptaken or bound by cancer cells to elicit beneficial PTT *in vivo* [18]. PTT could also be deployed using NP targeted to cancers during procedures involving open body cavities, such as exploratory surgical debulking of disseminated colorectal or ovarian cancer confined to the peritoneum. These maladies often present as many disseminated micro-tumors covering the surface of the organs within the *abdomen* and an open procedure would allow for direct application of light without the impediment of skin, muscle, and fat.

In many facets of medicine, polymers are used, often as tissue scaffolds or for encapsulating agents for selective delivery. Most medical polymers are relatively electrically inert, and the current work focuses on the class of conjugated polymers which inherently facilitate the mobility of electrons along the backbone of the polymer. Absorption of light stimulates the generation of electron-hole pairs which can propagate along the polymer chain. When these polymers are condensed into a nanoparticle, absorption of the light promotes electron-hole pair formation followed by rapid recombination and generation of heat due to confinement of the charges within the nanoparticle. Although conjugated polymer nanoparticles have been used as PTT agents previously, one of the challenges is the development of polymers with tunable absorption peaks. Many of the classic conjugated polymers that have been evaluated previously for PTT have one broad peak covering large portions of the infrared

region [4–6]. Alternatively, conjugated polymers designed with delocalized pi-electrons due to the presence of alternating electron rich (donor, D) and electron-poor (acceptor, A) repeat units take advantage of the potential to selectively “tune” their optical absorption [19,20].

Our group has previously published on the use of poly[4,4-bis(2-ethylhexyl)-cyclopenta[2,1-b;3,4-b']dithiophene-2,6-diyl-alt-2,1,3-benzoselenadiazole-4,7-diyl] (PCPDTBSe) nanoparticles (spherical, tubular, or fiber-shaped) as photothermal agents for treatment of cancer *in vitro* [19,20]. See Scheme 1 for the structure of the PCPDTBSe polymer. One feature that is inherently unique to PCPDTBSe is that the polymer has two optical absorption peaks; one between 400 and 500nm and the other centered near 750nm [19,22]. Although the presence of both peaks is not completely understood, they are believed to be from: (1) charge transfer between donor and acceptor units; (2) a pi–pi* transition centered on the donor unit; or (3) reorganization of molecular orbitals [22]. We have previously shown that 800nm laser stimulation of these NP can produce temperatures up to 85°C dependent on the NP concentration and laser parameters. In addition, the NP were shown to be able to reproducibly generate heat over multiple heating/cooling cycles with no loss of efficiency [19]. Incubation of cancer cells with PCPDTBSe NPs with a maximum concentration of 100µg/ml for 24 hours did not cause toxicity in absence of laser irradiation [21]. Stimulation of PCPDTBSe NP, incubated with cancer cells, with 800 nm laser at fluence of 229.2J/cm² revealed that concentrations above 30µg/ml resulted in 40–60% reduction in viable cells [21].

In the current study, we investigated the heat generation produced by stimulating PCPDTBSe polymer NPs with 450 and 800 nm lasers independently as well as simultaneously. The rationale for seeking dual stimulation of the nanoparticles using both blue and red light irradiation is that both regions of the optical spectrum have been shown to be beneficial for treating surface diseases, such as healing skin lesions and reducing acne vulgaris [23–27]. As the previous literature has already demonstrated the safety and efficacy of using blue and infrared light together for healing of surface diseases/wounds, it may be assumed safe to employ two wavelengths inducing photothermal ablation using PCPDTBSe NP. To date, there are only a handful of articles describing the coupling of multiple wavelengths with nanoparticles, although there is an excellent review of nanoparticle-based phototherapy for wound repair [28]. One example has explored the potential of graphene oxide to be stimulated to generate heat for enhanced stimulation of a photosensitizer agent [29]. However, no one has yet demonstrated heat generation from nanoparticles using stimulation of both optical absorption peaks simultaneously. Furthermore, there are very few nanoparticles available with dual absorption peaks; for example, metal nanorods which have optical absorption due to the transverse (weak) and longitudinal (strong) electron oscillation modes [30]. In contrast, both absorption peaks for PCPDTBSe NP exhibit strong, and nearly equivalent, optical density.

Blue light between 415 and 480 nm has been previously evaluated for inhibiting fibrosis by reducing the migration speed and proliferation of fibroblasts *in vitro* [31] as well as *in vivo* [24,32]. Specifically 420 nm was found to inhibit fibroblast proliferation, prevent their differentiation into myofibroblasts and reduce cellular contraction, and thus in turn reduce fibrosis *in vitro* [31]. When blue light wavelengths (400–500 nm) were tested for their

therapeutic/toxic effects *in vitro*, progressively shorter wavelengths of blue light, 410–420 nm, induced oxidative stress, leading to the conclusion that they are toxic to cells [33]. However, 453 and 480 nm light did not result in the same negative consequences, even when higher fluences (up to 30 J/cm²) were evaluated [33]. Blue light is often used for photodynamic therapy, wherein agents that absorb the incident radiation are stimulated to impart reactive oxygen species to damage cells, and this has been demonstrated *in vitro* in a number of articles [9,34,35]. Blue light has also been used without chemical sensitizers to treat tumors. For example, Ohara et al. used low intensity 470 nm light applied daily to inhibit the formation of skin tumors, reduce tumor size and prolong the survival of mice [23]. However, one of the limitations of using blue light for therapies, including PTT, is that shorter wavelengths do not penetrate as deeply into tissue as do longer wavelengths, mainly due to increased scattering of shorter wavelengths [31,36]. Scattering of blue light impedes the clinical utility of using blue light to stimulate nanoparticles for the treatment of deeply embedded tumors; however, there are many surface maladies, including skin cancer, infected dermal wounds, and esophageal or colorectal surface malignancies where application of blue light would effectively penetrate the tissue to stimulate nanoparticles for PTT. This would be especially valuable in treating diseases where the target tissue is readily accessible for receiving light stimulation, such as in open abdominal procedures for treating disseminated colorectal or ovarian cancer. One of the major challenges with cancer recovery is repair of the surrounding tissue after thermal injury, and blue and red light have been previously shown to be beneficial for healing of non-malignant tissue. Therefore, we sought to explore what the impact of using both blue and red light for simultaneous photothermal stimulation of PCPDTBSe NP might be on a cell line that is often used in animal models of disseminated colorectal cancer.

Similar to the evaluations using blue light for photo-biomodulation, red and NIR light (660–830 nm) have been explored to understand the positive effects of longer wavelengths. Low level laser therapy (LLLT) using 810 nm light has been shown to affect levels of reactive oxygen species (ROS), and in turn modify the cell survival transcription factor NF-κB in neuronal cells, *in vitro*, using 30 J/cm² or less. Calcium, adenosine triphosphate (ATP), mitochondrial membrane potential (MMP), and nitric oxide were also shown to be up- or down-regulated depending upon the fluence [37]. *In vitro* evaluation of photobiomodulation has been proposed to be beneficial by operating on cellular metabolism and energy pathways, with cytochrome c, porphyrins, and heme proteins available as principal light absorbers [37,38]. Low fluence (5 J/cm²) of 830 nm light has also been demonstrated to promote fibroblastic capacity for wound healing, as evaluated in an *in vitro* scratch assay model, by increasing nitric oxide, leading to a decrease in pro-inflammatory cytokines such as interleukin (IL-1B) and tumor necrosis factor (TNF-α) [39]. Increasingly more studies are evaluating the benefits of light at opposite ends of the visible spectrum (blue and red/NIR) for treating dermal wounds and cancer [23,32,39,40]. In non-hyperthermia models, 480 nm has been proposed for treating infected wounds and 730 nm light for assisting with healing of the wound [26]. Both blue and NIR have been used individually and together in an effort to reduce bacterial load and it was demonstrated that a synergy between the two wavelengths occurs, leading to greater reduction of bacterial load [41,42]. The onset of using dual

wavelengths for clinical treatment prompted our laboratory to explore this avenue in an effort to capitalize on the dual absorption present in PCPDTBSe polymer nanoparticles.

MATERIALS AND METHODS

Cell Lines and Reagents

CT26 mouse colorectal adenocarcinoma cells were purchased from American Type Culture Collection (Manassas, VA). The cells were cultured according to standard protocols using RPMI 1,640 medium, supplemented with 10% fetal calf serum and penicillin and streptomycin.

4H-Cyclopenta-[1,2-b:5,4-b']dithiophene was purchased from Tokyo Chemical Industry Co. Ltd. America (Portland, OR). 4,4-Bis(2-ethylhexyl)-2,6-bis(trimethylstannyl)-4H-cyclopenta-[2,1-b:3,4-b']dithiophene, and 4,7-dibromo-2,1,3-benzoselenadiazole were synthesized according to literature procedures [20,43]. Poly [4,4-bis(2-ethylhexyl)-cyclopenta[2,1-b:3,4-b']dithiophene-2,6-diyl-alt-2,1,3-benzoselenadiazole-4,7-diyl] (PCPDTBSe) was synthesized using a Stille-coupling step-growth polymerization as reported in the literature [19].

Intralipid® 20%, bovine hemoglobin, sodium alginate, and Pluronic® F-127 were purchased from Sigma–Aldrich (St. Louis, MO).

Synthesis of Polymeric Nanoparticles

PCPDTBSe (1 ml, 2 mg/ml) dissolved in tetrahydrofuran (THF) was added to Pluronic® F-127 (8 ml, 1 mg/ml) under constant horn sonication (20% amplitude, 1 min) with Branson Digital Sonifier fitted with a microtip. After sonication, the NPs were autoclaved to remove any organic solvents and sterilize the particles. The NP solution was centrifuged at 4842 RCF for 30 minutes to isolate bigger NPs and any other aggregates, and the supernatant was further centrifuged at 16,873 RCF for 4 hours to obtain uniform, small NPs. These NPs were further washed with ultrapure deionized water and centrifuged further (16,873 RCF, 4 hours) to remove excess Pluronic® F-127.

Characterization of Nanoparticles

Once synthesized, NP were characterized for their size, colloidal stability, morphology, and optical properties. Particle size and zeta potential were measured using a Malvern Instruments Zetasizer Nano-ZS90 dynamic light scattering (DLS) system. UV-Vis absorption of NP in deionized water was evaluated using a Beckman Coulter DU® 730 Life Sciences UV-vis spectrophotometer. The PCPDTBSe NP solution was prepared at dilute concentration, placed onto Formvar® coated copper grids, the water evaporated and imaged without negative staining using a Tecnai™ Spirit transmission electron microscope (FEI, Hillsboro, OR).

Heat Generation With Nanoparticles

Two separate lasers from K-Laser, USA were used: the K-Cube diode laser was programed to emit 800 nm light, and a prototype K-Cube laser with a 450 nm diode was also used. Both

sources were programed for continuous wave emission for 60 seconds. The laser beams were configured to overlap on the same 1 cm diameter spot, under which the test samples described below were placed. Two hundred microliters of PBS containing 0, 5, 10, 25, 50, and 100 µg/ml of PCPDTBSe nanoparticles were added to wells in a 48-well plate. Solutions were exposed to 450 or 800 nm light, or both delivered simultaneously, at power densities of 1.91 and 3.82 W/cm² for 60 seconds, for fluences of 114.6 and 229.2 J/cm². When 450 and 800 nm were applied simultaneously at 3.82 W/cm² the combined fluences was 458.4 J/cm². Solution temperatures were measured immediately before and after laser exposure using a wire thermocouple.

Determining the Power Loss Through Tissue Phantoms

To determine the loss of power for 800 nm and 450 nm wavelengths through tissue, tissue phantoms were prepared using 1% alginate, 0.5% Intralipid® 20% (a known optical scattering agent), 0.08% bovine hemoglobin, and crosslinked for 2 hours in 50 mM CaCl₂. Phantom thickness was determined using calipers. Phantoms with increasing thickness were placed between the laser source and an OPHIR® brand thermal power meter. Either 450 or 800 nm wavelengths were separately employed at 1 W. The laser beam was positioned with a 1 cm spot size over the power meter optical sensor. Phantoms were placed on a thin glass coverslip over the optical sensor and the distance from the laser aperture to the height of the phantom was fixed for all samples.

NP Association With CT26 at Different Incubation Times Followed by PTA

CT26 cells were seeded in triplicate in a 48-well plate at 20,000 cells/well using RPMI 1640 media (10% FBS, 1% P/S, and 1% L-glut). Purified PCPDTBSe NP were sterilized by autoclaving and a solution at a concentration of 100 µg/ml in cell culture media was prepared. Next, media was removed from the cells and 200 µl of NP containing media was added to each well and incubated for 30, 60, 120, or 240 minutes at 37°C. Controls included cells in media (no nanoparticles) with no laser irradiation and cells in media with laser irradiation. After each incubation time, the NP solution was removed from each well, and collected in separate vials. After removal of NP solution, cells were washed with 500 µl of PBS twice. All of the wash solution was also collected with the corresponding NP solutions removed from the cells. These solutions were analyzed using UV-Vis spectrophotometry to determine the concentration of NP using a known concentration calibration curve.

Next, 200 µl of PBS was added to each well and the plates were returned to the incubator for 30 minutes prior to NIR laser irradiation (800 nm, 229.2 J/cm²) for PTA. After laser irradiation 1 ml of fresh cell culture media was added to each well and the cells were incubated at 37°C for 24 hours before conducting a cell viability assay, using CellTiter96® from Promega according to the manufacturer's instructions and following similar procedures described by Robinson et al., Zhou et al., and also Burke et al. [44–46].

Photothermal Ablation Studies

CT26 cells were plated in triplicate at a density of either 40,000 or 20,000 cells/well in 48-well plates and incubated overnight at 37°C to facilitate cell adherence. Sterile tissue culture plates of this type may lead to laser light scatter, especially if condensation occurs of the

plate lid. To circumvent this problem, fresh, sterile lids with no condensation were used during laser irradiation. Purified PCPDTBSe NP were sterilized by autoclaving prior to *in vitro* use. Immediately prior to photothermal treatment, the media was removed from the cells and replaced with 200 μ l of PBS containing 0, 5, 10, 25, 50, or 100 μ g/ml of PCPDTBSe nanoparticles. The plates were returned to the incubator to return to 37°C and were maintained at 37°C during laser exposure by placing them on top of a warm water bottle. Cells were exposed to 450 or 800 nm light, or both delivered simultaneously, at power densities of 1.91 and 3.82 W/cm² for 60 seconds, for fluences of 114.6 and 229.2 J/cm². When 450 and 800 nm were applied simultaneously at 3.82 W/cm² the combined fluence was 458.4 J/cm². Next, cells were washed with 1×PBS to remove PCPDTBSe nanoparticles and returned to the incubator for 24 hours post-irradiation with addition of fresh media to each well. Cell viability was tested using the CellTiter96® assay from Promega (Madison, WI) according to the manufacturer's instructions.

Statistical Analysis

A two-way ANOVA with a *post-hoc* Tukey's test with a 95% confidence interval was used to evaluate the statistical differences observed in the heat generation when 450, 800, or 450 and 800 nm wavelengths together were used. Statistical analysis for *in vitro* PTA was performed using two-way ANOVA with a *post-hoc* Tukey's test with 95% confidence interval and value of $\alpha = 0.05$.

RESULTS

NP Characterization

The PCPDTBSe polymer dissolved in THF has absorption peaks in the blue and NIR regions at 420 and 750 nm, respectively, (Fig. 1). Both absorption peaks broaden slightly upon formation of NP and overlap the 450 and 800 nm excitation wavelengths of the laser sources. In addition, the 420 nm peak intensity increases upon the formation of a nanoparticle, compared to the intensity of the polymer only. As shown in Figure 2, the hydrodynamic diameter and zeta potential for PCPDTBSe NP were found to have means of 80 nm and -12.5 mV, respectively, with polydispersity index of 0.27 -0.01 as determined using dynamic light scattering. There appear to be two different populations of nanoparticles. This is due to the nature of nanoparticle formation, which results in three populations of nanoparticles, big, small, and very small. The nano-particles used in this work focus on the use of small nanoparticles; however, during purification *via* centrifuging a portion of the very small nanoparticle remain. The TEM image also shows the presence of some small nanoparticles. The zeta potential is a measure of the electrokinetic surface potential of the nanoparticle. The Pluronic coating may not uniformly cover the surface of the nanoparticles, and gaps in the coating are most likely contributing to the broad profile of the zeta potential. The zeta potential for Pluronic alone was found to be fairly neutral. Polymer nanoparticles were also made without any Pluronic coating and the zeta potential was found to be strongly negative. We believe that the partial coating with neutral Pluronic on the highly negative PCPDTBSe polymer is contributing to the non-Gaussian curve of zeta potential. Both the bimodal size data and non-uniform zeta potential data could be modified by further purification of the nanoparticles to for better size selection control and uniform

surface coating. The moderate negative charge indicates that the nanoparticles may be considered mildly anionic with adequate stability. Their anionic zeta potential may also impede them from interacting closely with cell membranes, which also have an anionic charge. The TEM image indicates spherical morphology (Fig. 1, inset).

Heat Generation With Nanoparticles

The heat generated by the PCPDTBSe NPs was evaluated when stimulated with blue (450 nm) and NIR (800 nm) light. Change in temperature was monitored at different NP concentrations (Fig. 3) and laser fluences of 114.6, 229.2, and 458.4 J/cm². There was no statistical difference for any nanoparticle concentration between 450 and 800 nm at 229.2 J/cm². Nor was there any difference between 450 and 800 nm together (combined fluence of 229.2 J/cm²) compared to 450 nm at 229.2 J/cm². There was no statistical difference between 450 or 800 nm independently at 114.6 J/cm² either. All other pairwise comparisons among groups were statistically different, including 450 and 800 nm applied simultaneously (combined fluence of 229.2 J/cm²) compared to 800 nm alone at 229.2 J/cm². The use of either 450 or 800 nm wavelengths at the same fluence produced similar temperature increases. For example, the highest temperature change achieved with independent irradiation of 450 or 800 nm was 46°C. However, when both lasers were combined, the temperature change was up to 65°C. The change in temperature when both the lasers were used simultaneously was higher than individual application, essentially because either the 114.6 or 229.2 J/cm² was delivered simultaneously using 450 and 800 nm and the total irradiance was doubled. It is important to point out that the change in temperature of deionized water containing no PCPDTBSe NP, upon laser irradiation was very small. With the highest laser fluence (229.2 J/cm² for individual or 458.4 J/cm² for dual), we observed changes in temperatures of 2.9 ± 0.7 , 1.3 ± 0.3 , and 2.7 ± 0.4 °C upon irradiation of water (no NP) with 800, 450, and 450 + 800 nm wavelengths.

Power Loss Through Tissue Phantoms

The penetration depth of either blue or infrared light in tissue is an important factor for realizing the potential of PCPDTBSe NP for PTA. Tissue phantoms, which incorporated a known absorbing agent (hemoglobin) and a known scattering agent (Intralipid® 20%) were utilized to visualize the trends of percent loss of power for either 450 or 800 nm light through increasing phantom thicknesses. The results, presented in Figure 4, confirm that the laser power is progressively attenuated with increasing phantom thickness, with 450 nm being more profoundly inhibited compared to 800 nm. For example, a 0.75 mm thick phantom reduces the transmitted power of 450 nm light by 79% compared to a 50% reduction of 800 nm light. Phantom thickness of 4 mm reduced the power of 450 nm light by 99% compared to 82% for 800 nm light.

NP Associated With CT26 Cells at Different Time Points Followed by PTA

To evaluate the association of PCPDTBSe NP with CT26 colorectal cancer cells, the cells were incubated with a concentration of 100 µg/ml of NP for 30, 60, 120, or 240 minutes at 37°C. Following incubation the cells were washed to remove NP that had not bound to, or been uptaken by the cells. As shown in supplementary Figure S1A, the maximum amount of non-functionalized PCPDTBSe NP associated with cells (either adhered to cells or

remaining on the tissue culture plastic) was 6.78 μg and the lowest amount was 1.51 μg . Cells incubated for 60 or 240 minutes had a statistically significant decrease in the amount of NP associated with the cells compared to cells incubated for 0 (immediate washing), 30 or 120 minutes. Following binding and washing, the cells were exposed to 800 nm light at 229.2 J/cm² to determine if there was any observable photothermal effect on the cells that might indicate the presence of nanoparticles, and induction of heat, leading to a decrease in cell viability. For binding time of 120 minutes the number of cells following laser irradiation was statistically the same as cells treated without exposure to laser stimulus or nanoparticles. For cells incubated with PCPDTBSe NP for 0, 30, or 60 minutes, there was a statistically significant increase the number of viable cells 24 hours following laser exposure, with a 150% increase in cells incubated for 30 minutes and washed before exposure to 800 nm light. However, PTA did occur in the subset of cells incubated with 100 $\mu\text{g}/\text{ml}$ (or a total mass amount of 20 μg) of PCPDTBSe NP for 240 minutes. There was a 25% reduction in the number of viable cells, and this result was statistically different from the controls.

Photothermal Ablation Studies

The effect of blue and NIR wavelengths on heat generation was evaluated for photothermal ablation in colorectal cancer cells at variable NP concentrations and laser fluences of 114.6, 229.2, and 458.4 J/cm², and the results are provided in Figure 5. At either 20,000 or 40,000 cells, application of either 114.6 or 229.2 J/cm² led to a progressive decrease in cell viability for NP concentrations above 25 $\mu\text{g}/\text{ml}$. The values listed above each bar correspond to the temperature increase above 37°C that the cells were expected to be subjected to during laser irradiation. The asterisks denote a statistically significant deviation ($P < 0.05$) from the control (0 $\mu\text{g}/\text{ml}$ NL [no laser]). The statistically significant decreases in cell viability appear to correspond with the significantly elevated temperatures imparted at high NP concentration. Notably, there is a statistically significant increase in cell viability when mild hyperthermia is induced using only 450 nm irradiation or 450 nm stimulation of the 5 $\mu\text{g}/\text{ml}$ PCPDTBSe NP concentration. A similar upward trend in cell viability was observed using 800 nm laser irradiation and 0 or 5 $\mu\text{g}/\text{ml}$ of NP for the lower cell density (Fig. 5B), regardless of whether 114.6 or 229.2 J/cm² was used. This is not an unexpected result as LLLT and/or the induction of mild hyperthermia have routinely been shown to have a stimulatory effect on cellular functions, sometimes leading to enhanced cell proliferation [47,48]. However, at the higher cell density (Fig. 5E), the higher fluence resulted in a slightly decreased cell viability for the 0 and 5 $\mu\text{g}/\text{ml}$ laser irradiated groups. It is not clear why the higher fluence results in the slight cell reduction, even with mild hyperthermia. However, the onset of highly reduced cell viability is observed at 10 $\mu\text{g}/\text{ml}$ with application of 229.2 J/cm² in the high cell density group. Using 800 nm laser irradiation there appears to be an enhanced PTA effect using the higher fluence and higher cell density, although the connection is not yet understood. Temperatures of sample solutions from Figure 3 indicate that cells in the presence of the 5 and 10 $\mu\text{g}/\text{ml}$ solutions would be subjected to temperatures significantly over the mild hyperthermia threshold of 45°C. However, as seen in Figure 5C and F, at either low or high cell density there was an increase in cell viability for the 0 and 5 $\mu\text{g}/\text{ml}$ groups and no change in the 10 $\mu\text{g}/\text{ml}$ group as compared to the control. This suggests that although dual stimulation of the PCPDTBSe NP with 450 and 800 nm light simultaneously generates significant heat, that there may be a protective effect, perhaps due

to the 450 nm wavelength, that minimizes, or allows cells to overcome, the rapidly delivered thermal damage. For cell densities the onset of very significant decreases in cell viability occurs at 25 $\mu\text{g/ml}$ with complete ablation at 50 $\mu\text{g/ml}$. One conclusion of this study is lower concentrations (between 25 and 50 $\mu\text{g/ml}$) of PCPDTBSe NP may be acceptable for complete PTA when both wavelengths are used.

DISCUSSION

Typical photothermal ablation therapies using NP are performed using NIR absorbing materials. In this study, we sought to explore dual-wavelength induced photo-thermal therapy using a unique NP material that can produce heat with laser irradiation at two separate wavelengths. PCPDTBSe NP developed in our lab have strong absorption at 420 and 750 nm which can easily be excited with 450 and 800 nm light to generate heat. The NP had comparable heat generation using either the 450 or the 800 nm wavelengths. When both wavelengths combined to stimulate the NP twice as much heat was generated. Initially, it was anticipated that stimulation of the two wavelengths simultaneously for heat generation would allow for a reduction in the laser power density and concentration of NP needed to achieve photothermal ablation. As shown in Figure 3, laser fluence correlates to temperature increases regardless of whether 450 or 800 nm light is used for stimulation of the PCPDTBSe NP. For example, using 50 $\mu\text{g/ml}$ and stimulation of PCPDTBSe NP with 114.6 J/cm^2 , either 450 or 800 nm light results in a temperature change of about 18°C. Blue and NIR light applied together, each at 114.6 J/cm^2 , for a total fluence of 229.2 J/cm^2 , results in a temperature change of about 32°C. The laser intensities here are higher than those explored in previous work using LLLT so that elevated hyperthermia for cell ablation will be induced using the PCPDTBSe NP. When mild hyperthermia was induced *via* laser irradiation alone or stimulation of the PCPDTBSe NP, a protective effect and substantial increase in the number of viable cells was seen, as shown in Figure 5. This effect was seen using either 450 or 800 nm alone, or when they were simultaneously employed. This result corroborates evidence from other researchers on the potential benefits of mild hyperthermia for facilitating wound healing [26,49,50]. Additionally, as demonstrated previously, blue light may further be able to enhance healing by stimulating intracellular chromophores [38]. The data from Figure 5C and F show that even at elevated temperatures (5 and 10 $\mu\text{g/ml}$ concentrations), the dual application of 450 and 800 nm seem to be offering a mechanism for cells to recover from thermal damage. Based on previous literature, the positive benefits of blue light combined with simultaneous application of red and infrared light, has been shown to support wound healing by upregulating cellular metabolism, possibly because of light absorption by cytochrome c [51,52]. Since clinical studies are moving toward the application of blue and infrared light together for healing, it seemed prudent for us to explore whether both wavelengths could be used simultaneously to stimulate PCPDTBSe NP for generation of heat and evaluate the results on a cell line frequently used in animal models.

Specifically because PCPDTBSe has two distinct peaks in the blue and near infrared, the current work has sought to explore whether the NP could be stimulated using either wavelength. The mechanism for heat generation in PCPDTBSe NP is the formation and recombination of electron and hole pairs; thus, the greater the distance between the electron

and hole, the more elastic energy, leading to stronger recombination, and potentially enhanced heat generation. We were hopeful that stimulation with one wavelength would generate sufficient heat to provide a more favorable environment for increasing the distance between electrons and holes, and hence lead to more heat being generated by the PCPDTBSe NP. Our results do not confirm that this is possible using a continuous wave laser, although short pulsed laser application might be able to generate this effect. Specifically a laser with a pulse duration much shorter than the thermal relaxation time of tissue might afford for high power densities to be utilized, thus allowing for greater penetration depth [53,54].

As shown in Figure 5, two thresholds need to be attained in order to induce sufficient cell killing: concentrations of PCPDTBSe NP above 25 $\mu\text{g/ml}$ are ideal, as are elevated laser fluences. Although no benefit for dual stimulation of the nanoparticles was seen for PTT, our work demonstrates that a new class of NP, built upon the platform of optically tunable donor-acceptor polymers, is available for effective PTT, using either blue or near infrared light. This may open the door for future clinical translation in treating a host of surface malignancies, since blue and infrared applied together is rapidly moving through clinical evaluation.

The nanoparticles described in this work are based on polymers, and we hypothesize that they should not degrade *in vivo*, and this is proposed for future exploration. Because the polymer itself is very hydrophobic the polymer chains prefer to remain self-assembled into nanoparticles. Our previous works specifically on PCPDTBSe NP have shown that the NP do not aggregate or precipitate in a variety of media, including high and low pH solutions [19,21]. They are also resistant to extreme temperatures including multiple heat/cool cycles *via* laser irradiation and autoclaving [19]. Additionally, a host of similar conjugated polymer nanoparticles have been developed over the past decade for fluorescent imaging and they have been evaluated in animal models and shown to be non-toxic *in vivo* [55–58]. PCPDTBSe NP should behave similarly and as they are inert, are expected to be cleared by the reticulo-endothelial system.

One of the challenges with PTT is the depth of penetration of certain wavelengths in tissue. As demonstrated in Figure 4, 450 nm light is substantially more impeded as the thickness of the tissue phantom is increased. Although the laser power is impeded for 800 nm also, a greater percentage of the power is transmitted through the phantoms, with 10% of the power still available for stimulation of nanoparticles at a depth of 6 mm. As both blue and NIR light may be strongly attenuated, as demonstrated by our tissue phantom results, the use of either wavelength, but most especially blue light, for the stimulation of NP, and subsequent generation of hyperthermia, would have the greatest clinical relevance in treating surface malignancies. Oral or dermal cancers are primary examples, as are other tissues which can be accessed *via* endoscopy (esophageal, stomach, colorectal). PTT could also be deployed using NP targeted to cancers during procedures involving open body cavities, such as exploratory surgical debulking of disseminated colorectal or ovarian cancer confined to the peritoneum. These maladies often present as many disseminated micro-tumors covering the surface of the organs within the *abdomen* and an open procedure would allow for direct application of light without the impediment of skin, muscle, and fat.

CONCLUSION

A novel polymer nanoparticle was explored for the generation of heat and subsequent photothermal ablation of colorectal cancer cells *via* stimulation with either blue, NIR, or both applied simultaneously. PCPDTBSe NP have excellent heating in both the blue and NIR range and demonstrated their effectiveness for use as PTT agents stimulated by either wavelength. Both 450 and 800 nm light was attenuated in the tissue phantoms, with blue light being more strongly impeded. Therefore, PTT using dual wavelength stimulation would be most suitable for surface malignancies. The combination of blue and NIR light is in the beginning stages of being explored for clinical applications, and a nanoparticle that would impart hyperthermia using either wavelength could help initiate new areas of research using NP capable of dual stimulation for healing cancer and treatment of soft tissue maladies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. UV-Vis and NIR absorption properties of PCPDTBSe polymer (dotted) as well as NP (solid) dissolved in THF and water, respectively. Spherical morphology of the NP was observed using transmission electron microscopy (inset). Of note is the increase in the intensity of the absorption peak at 450 nm upon nanoparticle formation.

Fig. 2.
(A) Histogram representation of nanoparticle size distribution. (B) Zeta potential measurement.

Fig. 3.

Heat generation of PCPDTBSe NP stimulated with blue (450 nm) and NIR (800 nm) light, either separately (114.6 J/cm²—closed triangles for 450 nm and closed square for 800 nm or 229.2 J/cm²—open triangles for 450 nm or open squares for 800 nm) or applied simultaneously (229.2 J/cm²—open diamonds or 458.4 J/cm²—closed diamonds). Simultaneous stimulation of the PCPDTBSe NP doubles the total laser fluence such that when 114.6 J/cm² is applied using both 450 and 800 nm light the total fluence for both wavelengths is 229.2 J/cm². Similarly, when both 450 and 800 nm light is applied at 229.2 J/cm² the combined fluence is 458.4 J/cm².

Fig. 4.

The percent decrease in total laser power when using either 450 nm (blue filled squares) or 800 nm (red filled diamonds) light, dependent upon the thickness of sodium alginate tissue phantoms doped with hemoglobin absorbing agent and Intralipid scattering agent.

Fig. 5.

Effective photothermal ablation was measured by evaluating the viability of CT26 mouse colorectal cancer cells exposed to increasing concentrations of PCPDTBSe NP and increasing laser fluences. NL represents “no laser.” For (A–C) 20,000 cells per group were used. For (D–F) 40,000 cells per group were used. (A, D) Application of 450 nm light only. (B, E) Stimulation with 800 nm light only. (C–F) Stimulation with 450 and 800 nm light applied to the same spot. Simultaneous stimulation of the PCPDTBSe NP doubles the total laser fluence such that when 114.6 J/cm² is applied using both 450 and 800 nm light the total fluence for both wavelengths is 229.2 J/cm². Similarly, when both 450 and 800 nm light are applied at 229.2 J/cm² the combined fluence is 458.4 J/cm². Groups which had a statistically significant increases or decreases in cell viability compared to the no nanoparticle, no laser control are marked with an asterisk. Also, for all groups exposed to laser irradiation, the change in temperatures (in °C) that correspond to the applied laser fluence have been added above the bar for reference.

Scheme 1.

Schematic of PCPDTBSe polymer where the segments responsible for electron donation and electron acceptance are denoted by a red D or A.