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Triple-responsive Expansile Nanogel for Tumor and Mitochondria Targeted Photosensitizer Delivery

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Abstract

A pH, thermal, and redox potential triple-responsive expansile nanogel system (TRN), which swells at acidic pH, temperature higher than its transition temperature, and reducing environment, has been developed. TRN quickly expands from 108 nm to over 1200 nm (in diameter), achieving more than 1000-fold size enlargement (in volume), within 2 h in a reducing environment at body temperature. Sigma-2 receptor targeting-ligand functionalized TRN can effectively target head and neck tumor, and help Pc 4 targeting mitochondria inside cancer cells to achieve enhanced photodynamic therapy efficacy.

Keywords

Stimuli-responsive; nanoparticle; tumor targeted; expandable; photodynamic therapy

1. Introduction

Photodynamic therapy (PDT) is a treatment procedure that uses a light to activate a photosensitizer (PS) to produce singlet oxygen for killing cancer cells or curing acne [1]. Since the onset of PS toxicity can only be triggered by the irradiation of light, PDT generally is considered as a safe alternative for chemotherapy in the treatment of cancer, and does not induce side effects. A lot of evidences indicate that PDT- induced apoptosis is due to the damage of mitochondria and suggest that mitochondria are the target for PDT [2]. Therefore, the efficacy of PS would be greatly enhanced if it can be delivered specifically to the mitochondria of cancer cells. In fact, research revealed that Pc 4, a silicon phthalocyanine PS, can spontaneously partition to mitochondria due to its high hydrophobicity, which

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makes it an ideal PS for maximizing PDT efficacy [3]. However, the clinical application of Pc 4 based PDT has not been widely accepted due to its poor water solubility and erratic tissue retention, especially in the skin which results in unwanted tissue damage upon the exposure to sunshine.

Over past decades, many types of nanoparticle carriers have been developed for targeted delivery of Pc 4 to tumor by taking advantage of the leaky vascular structure in tumor tissue through so called enhanced permeability and retention (EPR) effect. Such systems include polymeric micelles, mesoporous silica nanoparticles, and gold nanoparticles, which can load hydrophobic Pc 4 through hydrophobic interaction [4–6]. Although with the help of various ligand-receptor interactions most nanoparticles achieved enhanced cellular uptake of Pc 4, there was occasional disconnection between the uptake of PS and their PDT efficacy. Higher uptake of PS did not result in better cell killing, possibly due to the fact that those encapsulated Pc 4 could not effectively escape from lysosome and then transfer to mitochondria [3, 7].

Therefore, we hypothesize that a nanocarrier which can escape from lysosome, quickly expand its size to release Pc 4 into cytosol would be able to deliver Pc 4 to mitochondria. Expansile nanoparticles (eNP), which can enlarge their size in response to pH, have been explored as drug carriers to control the drug release at targeted sites and achieved enhanced therapeutic effect [8–11]. Nanogels fabricated from pyridyl disulfide containing polymers have been applied in various drug delivery systems due to their easy functionalization [12, 13]. Recently, our group reported a multicompartiment nanogel made of poly[(2-(pyridin-2-yl)disulfanyl)-co-[poly(ethylene glycol)]] (PDA-PEG) polymer, which showed self-expanding property in reducing environment and size increasing from 115 nm to 262 nm in 5 h [14]. In addition, the release of its payload was dependent on its environmental pH and redox potential. The abundance of pyridine segments endowed the proton sponge effect of the polymer and helped its escaping from lysosome.

To extend the sensitiveness of the nanogel to temperature, a thermal responsive polymer, poly (N-Isopropyl methacrylamide) (PNiPMA), was incorporated into the PDA-PEG by free radical polymerization to yield a pH, redox potential, and thermal triple-responsive polymer PDA-PEG-PNiPMA as described in Figure 1A. A triple-responsive nanogel (TRN) was fabricated with the help of predesigned amounts of tris(2-carboxyethyl)phosphine (TCEP).

2. Materials and Methods

2.1. Materials

Aldrithiol-2 and Silica gel (Spherical, 100 μm) were purchased from Tokyo Chemical Industry Co., LTD (Harborage Street, Portland, OR). 2-mercaptoethanol, DL-dithiothreitol (DTT), tris(2-carboxyethyl)phosphine (TCEP), 2, 2-Azobisisobutyronitrile (AIBN) and Poly(ethylene glycol)methacrylate ($M_n = 360$ Da) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Penicillin (10,000 U/mL), streptomycin (10,000 $\mu\text{g/mL}$), 0.25% trypsin-EDTA, Dulbecco's Modified Eagle Medium (with L-glutamine) and fetal bovine serum (FBS) were obtained from American Type Culture Collection (ATCC, Manassas, VA). 2,4,6-Trinitrobenzene sulfonic acid (TNBSA) was purchased from Thermo

Scientific. Silicon phthalocyanine (Pc 4) was acquired from NCI (NSC 676418). All the other solvents used in this research were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) and used without further purification unless otherwise noted.

2.2. Synthesis of PDA-PEG-PNiPMA polymer

PDA-PEG-PNiPMA polymer was synthesized by free radical polymerization as shown in Figure 1A. PDA monomer was prepared following our previously reported method [14]. Typically, PDA (241.3 mg, 1 mmol), Poly(ethylene glycol)methacrylate ($M_n = 360$ Da) (360 mg, 1 mmol) and *N*-isopropylmethacrylamide (PNiPMA, 63.6 mg, 0.5 mmol) were dissolved in 10 mL degassed anisole. 2, 2-azobisisobutyronitrile (AIBN, 14 mg, 0.085 mmol) in 1 mL degassed anisole was then added, and the reaction mixture was stirred for 24 hours at 65 °C. The final product was precipitated (3×) in ice cold ether and dried for 48 hours in vacuum. The structure of PDA-PEG-PNiPMA was confirmed by $^1\text{H-NMR}$ (Figure S1A). Gel permeation chromatography (viscotek GPCmax VE 2001 GPC solvent/sample module, Viscotek VE 3580 RI detector and 270 Dual Detector) using THF as mobile phase was employed to characterize the polymer and found PDA-PEG-PNiPMA has an absolute molecular weight of 27,557 Da (M_w) and polydispersity (PDI: 1.35) (Figure S1B). For the quantification of side chain functionality, PDA-PEG-PNiPMA (1.0 mg/mL) was dissolved in DMSO and incubated with dithiothreitol (DTT, 10 mM) for 1 hour at room temperature, and then the amount of 2-pyridinethione released was quantified through UV-Vis spectrophotometer at $\lambda = 375$ nm (ϵ , molar absorption coefficient = $8080 \text{ M}^{-1}\text{cm}^{-1}$).

2.3. Synthesis of MBA-PDA-PEG-PNiPMA

PDA-PEG-PNiPMA was further modified with sigma-2 receptor targeting motif (4-Methoxybenzoic acid (MBA)). Briefly, cysteamine (0.404 mg, 20% PDA function groups) in 500 μL DMSO was added dropwise into 20 mg PDA-PEG-PNiPMA in 500 μL DMSO and the reaction mixture was kept at room temperature overnight. After overnight reaction, the product was dialyzed towards DMSO using Spectra/Por® dialysis tube (MWCO: 1000 Da). The concentration of amine group in the polymer after dialysis was quantified by TNBSA assay. MBA (1.66 mg in 100 μL DMSO, 50% PDA function group) was firstly activated by EDC (4.2 mg in 50 μL DMSO) and NHS (2.5 mg in 50 μL DMSO), and then added into 20 mg cysteamine modified PDA-PEG-PNiPMA polymer. The reaction was kept overnight at room temperature. The amine concentration in PDA-PEG-PNiPMA polymer after MBA conjugation was also quantified by TNBSA assay to determine the MBA conjugation efficiency. TNBSA assay result revealed that 16% MBA (to PDA ratio) has been successfully conjugated to the polymer.

2.4. TRN nanogel fabrication

Briefly, PDA-PEG-PNiPMA and MBA-PDA-PEG-PNiPMA were mixed to obtain different MBA ligand density for TRN. The polymer mixture (5 mg) was dissolved in 300 μL DMSO. Pc 4 (250 μg) was dissolved in 100 μL DMSO and then added into the polymer mixture. For the fabrication of TRN with 30% cross-linking density, tris (2-carboxyethyl) phosphine hydrochloride (TCEP, 0.384 mg in 20 μL DMSO) was added to the above mixture. The reaction mixture was equilibrated for 15 min and then dropped into 4 mL ddH₂O under

stirring and kept stirring for aerial oxidation overnight. After the oxidation, the nanogel was then dialyzed towards PBS of pH 7.4 (10 mM) for 10 h to remove unreacted TCEP, MBA and organic solvent. Finally, the nanogel was filtered (0.45 μ m syringe filter) and stored in 4 °C. The morphology, size distribution and the surface charge (ζ -potential), of the nanogel were determined by a Hitachi H8000 transmission electron microscopy (TEM) and dynamic light scattering (DLS) as previously reported [15].

2.5. Pc 4 nanogel drug concentration determination and release kinetic assay

Pc 4 shows maximum absorbance at peak 670 nm. Therefore, Pc 4 concentration in nanogels was measured at 670 nm by UV-Vis spectroscopy. Pc 4 nanogel 2 μ L was diluted with 980 μ L DMSO (diluted 50 $\times$) and measured by UV-Vis. Drug concentration was then calculated by calibration curve. Pc 4-TRN was suspended in PBS of pH 7.4 (10 mM) at the final concentration of 10 μ g/mL. To mimic the drug release process of Pc 4-TRN during blood circulation and inside lysosome, Pc 4-TRN was dialyzed towards 40 mL PBS (pH 7.4, 10 mM, 1% Tween 80) and acetate buffer (pH 5.0, 1% Tween 80) at 37 °C, respectively. At pre-determined time intervals, 1 mL dialysis buffer was removed and replaced with 1 mL fresh buffer. The samples were stored at -20 °C till measurement. After collecting all samples, 100 μ L each sample was loaded into 96 well plate (Costar, black, clear bottom) and Pc 4 concentration was quantified by fluorescence (Ex 610 nm, Em 680 nm, SpectraMax M2 Multi-Mode Microplate Reader). A calibration curve was constructed by adding known concentrations of Pc 4 to PBS pH 7.4 (10 mM, 1% Tween 80) acetate buffer (pH 5.0, 1% Tween 80). To simulate the process of Pc 4-TRN escaping from lysosome and transfer to cytosol, the pH of releasing buffer was adjusted to 5.0 at first 2 h. After that, the pH of the releasing buffer was adjusted to 7.2 and kept at this pH for remaining experiment, GSH (final concentration was 10mM) was added into the buffer at the same time. As a control group, no GSH was added into buffer; however, the pH of the buffer was also adjusted to 7.2 and kept at this pH for the remaining experiment. At pre-determined time intervals, samples were retreated and Pc4 concentration was measured by fluorescence as previously described.

2.6. Cellular uptake of TRN observed by confocal microscopy

UMSCC22A cells (200,000 cells/dish) were cultured on 35 mm² Petri dishes (MatTek, MA, USA) for overnight. The media were replaced with fresh media containing 16% MBA-Pc 4-TRN, Pc 4-TRN, and free Pc 4 (equivalent to 200 nM Pc 4). After 4 or 20 h of incubation under a humidified atmosphere of 95/5% air/CO₂, cells were washed by PBS(3 $\times$), fixed with formaldehyde (4.5% in PBS) and stained with Hoechst 33342 (final concentration 1 μ g/mL). Then cells were analyzed under a confocal microscope (LSM 510, Carl-Zeiss Inc.).

2.7. Quantification of intracellular Pc 4 amount

UMSCC22A cells (100,000 cells/well) were cultured on 24-well plate for overnight. Culture media were replaced with fresh media containing free Pc4 drug, MBA (16, 8, 4, 1, and 0%) Pc 4-TRN nanogels (equivalent to 200 nM Pc 4). After incubation under a humidified atmosphere of 95/5% air/CO₂ for 4 and 20 h, respectively, cells were washed by PBS(3 $\times$) and lysed in 0.5% SDS. Cell lysates were collected and Pc 4 concentration was quantified by fluorescence (Ex 610 nm, Em 680 nm). Total protein concentrations of cell lysates were

measured by BCA kit following manufacturer's instruction (Thermo Fisher Scientific). The ratio between Pc 4 and protein was used to evaluate the ability of UMSCC22A cells take up Pc 4 and Pc 4-TRN of different MBA density.

2.8. Immunohistochemistry analysis

Human tissues were collected under IRB protocol approved by the Institutional Review Board of the Medical University of South Carolina. FFPE sections of human head and neck tumor tissue microarray were de-paraffinized in xylene, rehydrated in alcohol, and processed as follows: The sections were incubated with target retrieval solution (Dako S2368) in a steamer (Oster CKSTSTMD5-W) for 45 min, 3% hydrogen peroxide solution for 10 min and protein block (Dako X0909) for 20 min at room temperature. After overnight incubation with sigma-2 antibody (Sigma HPA002877) in a humid chamber at 4 °C, biotinylated anti-rabbit secondary antibody (Vector, PK-6101) and ABC reagent (Vector, PK-6101) was added for 30 min. Immunocomplexes of horseradish peroxidase were visualized by DAB (Dako, K3468) reaction, and sections were counterstained with hematoxylin before mounting.

2.9. Western Blot Analysis

UMSCC22A cell extracts were prepared in ice-cold RIPA lysis buffer (150 mM NaCl, 1 mM EGTA, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1% NP40, 50 mM Tris-Cl, pH 7.4) supplemented with a cocktail of protease inhibitors (Roche Diagnostics) and centrifuged. Proteins (75 µg) in sample buffer (Invitrogen) supplemented with 10% SDS and 10% β-mercaptoethanol were resolved on NuPAGE® Tris-bis 4%–12% polyacrylamide gels (Invitrogen). Proteins were transferred to PVDF membranes (EMD Millipore) and probed with anti-sigma-2 (PGRMC1) (1:1000) (Cell Signaling). Membranes were developed by the Enhanced Chemiluminescence Detection System (Thermo Fisher Scientific), and band intensities were quantified using a Carestream 4000 PRO image station (Woodbridge, CT).

2.10. Sub-cellular co-localization of TRN

UMSCC22A cells (150,000 cells/dish) were cultured onto glass-bottomed MatTek dishes and incubated with MBA-Pc 4-TRNs at indicated times. Before imaging, medium was changed to fresh medium supplemented with Insulin-Transferrin-Selenium-X (ITX) reagent [insulin (10 µg/ml), transferrin (5.5 µg/ml), selenium (6.7 ng/ml), ethanolamine (0.2 mg/ml)] (Gibco) but omitting FBS. To assess co-localization of nanoparticles with mitochondria, cells were loaded with 500 nM tetramethylrhodamine methylester (TMRM). Medium was then changed with fresh medium containing 50 nM TMRM. To assess co-localization of MBA-Pc 4-TRNs with lysosomes, cells were loaded with 500 nM LysoTracker Green (LTG). Dishes were placed in an environmental chamber at 37 °C on the stage of a Zeiss LSM 510 laser scanning confocal microscope (Zeiss, Thornwood, NY). A 63 X N.A. 1.4 oil immersion planapochromat objective was used for all experiments. LTG, TMRM and Pc 4 fluorescence was imaged using 488 nm excitation/500–530-nm emission, 543 nm excitation/565–615 nm emission and 633 nm excitation/650–710 nm emission, respectively. ImageJ software was used to post-process the images and calculate the co-localization coefficients.

2.11. Photodynamic therapy

Cell cultures were incubated with 200 nM of MBA-Pc 4-TRN and Pc 4-TRN for 20 h before exposure to 200 mJ/cm² red light (670 nm) from an Intense-HPD 7404 diode laser (North Brunswick, NJ). After exposure to red light, cells were incubated for various periods of time prior to analysis.

2.12. Assessment of Cell Death after PDT

Cell death was assessed by propidium iodide (PI) fluorometry using a multi-well fluorescence plate reader, as previously described [16]. Human head and neck cancer cells (UMSCC22A) were plated on 96-well plates (15,000 cells/well) in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (complete culture medium) in a humidified 37 °C incubator at 5% CO₂/95% air. Subsequently, cells were incubated with MBA-Pc 4-TRN and Pc 4-TRN (200 nM) for 20 h. Before exposure to light, cells were changed to fresh medium supplemented with PI (30 µM) and Insulin-Transferrin-Selenium-X (ITX) reagent [insulin (10 µg/ml), transferrin (5.5 µg/ml), selenium (6.7 ng/ml), ethanolamine (0.2 mg/ml)] (Gibco) but omitting FBS. PI fluorescence was measured at frequent intervals using 530 nm excitation (25 nm band pass) and 620 nm emission (40 nm band pass) filters. Between measurements, microtiter plates were placed in a 37 °C incubator. At the end of the experiment, digitonin (200 µM) was added to each well to permeabilize all cells and label all nuclei with PI. Cell viability determined by PI fluorometry is essentially the same as cell viability determined by trypan blue exclusion [16].

2.13. Biodistribution of MBA-Pc 4-TRN

All animal experiments followed the protocols approved by the MUSC Institutional Animal Care and Use Committee (IACUC). Head and neck tumor xenografts were created with UMSCC22A cells (3×10^6 cells/mouse) in male athymic *Nu/J* mice (6 weeks old, inbred homozygous) (Jackson Labs). Once tumor volumes reached 50–150 mm³ measured with a caliper, mice were administered with MBA-Pc 4-TRN and free Pc 4 (1 mg/kg Pc 4) in PBS through the tail vein. Fluorescence images were taken with a Maestro 2 *in vivo* imaging system 72 h after dosing. Subsequently, mice were sacrificed at 96 h post-injection. Liver, spleen, heart, kidneys, lungs, and the tumor were collected and imaged.

3. Results

PNiPMA is a polymer which undergoes phase transition when the environmental temperature passing through its Low Critical Solution Temperature (LCST, around 43 °C), soluble in water at temperature lower than LCST while becoming hydrophobic at temperature higher than its LCST [17]. Nano/micro-particles containing PNiPMA shrink when the environment temperature is higher than its LCST [18, 19]. To investigate the effect of crosslinking density (CD) of TRN on its transition temperature, the transmittance of TRN was recorded during the course of temperature increase. Transmittance measurement revealed that the addition of PNiPMA did endow the temperature sensitivity to the nanogel: TRN nanogel suspension decreased its transmittance and appeared cloudy at high temperature (Figure 1B). The transition temperature of TRN shifted from 30.5 °C to 47 °C

as its CD increased from 20 to 40%, while no transition was recorded for TRN with 80% CD or higher. Therefore, TRNs with different transition temperatures can be attained by simply tuning CD during nanoparticle fabrication process.

Using the fabrication protocol described above, Pc 4 loaded TRN with a transition temperature slightly higher than body temperature can be easily produced from PDA-PEG-PNIPMA with 30% CD. Compared with its counterpart fabricated from PDA-PEG polymer, the loading efficiency of Pc 4 increased from 13 to 40% for TRN, which maybe due to the newly formed PNIPMA layer served as a buffer zone between the hydrophobic PDA and the hydrophilic PEG. To investigate how the TRN responds to the changes in temperature, redox potential, and pH after the loading of Pc 4, the sizes and morphologies of the TRN were measured with dynamic light scattering (DLS) and observed with transmittance electron microscopy (TEM), respectively [15]. The size of the TRN was 108.1 ± 11.1 nm with a PDI of 0.163 (Figure 2A). Zeta sizer found that TRN carried slightly negative surface charge (-5.62 ± 1.40 mV). TEM revealed that TRNs were spherical (Figure 2E). TRN itself was stable in PBS and culture medium containing 10% FBS (Figure S2), and no obvious size change was observed after 3 days of incubation. In contrast to its PDA-PEG di-copolymer nanogel counterpart, which kept constant size in the whole tested temperature range, TRN with 30% CD dramatically increased its size at temperature higher than 39 °C (Figure 2A). It is worth noting that the size enlargement in response to the temperature increase is totally different from other PNIPMA containing particles, which shrink upon environment temperature higher than their LCSTs [18, 19]. Figure 2A also showed that the addition of Pc 4 slightly decreased the transition temperature of TRN from 44 °C to 39 °C. The enlarged size of TRN shown in Figure 2F also evidenced the thermal expanding property of TRN.

To examine the redox potential effect on the transition temperature of TRN, 10 mM DTT was added during the heating process. To eliminate the possible effect caused by the intra-particle crosslinking of TRN after DTT treatment shown in Figure 2B, EDTA was added. Figure 2B revealed that the addition of 10 mM DTT/EDTA further decreased the transition temperature of TRN from 39 °C to 36 °C. It is known that cytosol has an elevated glutathione level (10 mM, much higher than that in the blood), which would result in the rapid intracellularly self-expanding of TRN at body temperature [20].

To further evaluate the sensitivity of TRN in response to reducing environment at body temperature, the size of TRNs suspended in media with or without 10 mM DTT/EDTA was monitored at 37 °C. As we expected, the size of TRN remained constant in PBS buffer (Figure 2C). To our surprise, under reducing environment, TRN swelled from 108 nm to 627 nm in 30 min and further expanded to larger than 1200 nm in less than 2 h (Figure 2C, 2G, and 2I), achieving more than 1000-fold size enlargement (in volume), which is more than 10-fold faster than its di-copolymer counterpart [14]. Besides its self-expansion in response to the increase of temperature and redox potential, DLS and TEM also revealed that the size of TRN was also sensitive to the change of pH. TRN instantly expanded its size from 108 nm to 203 nm upon the decrease of pH from 7.4 to 5.0, and then further increased to 360 nm in 16 h (Figure 2D and 2H).

To verify that the stimuli triggered size enlargement of TRN will result in faster release of Pc 4, drug release assay was carried out in pH 7.4 and 5.0 buffers to mimic the extracellular and lysosomal environments, respectively. Figure S3A showed that TRN released only 13.6% of Pc 4 in pH 7.4 buffer over 3 days of incubation, indicating that TRN is a stable carrier during the circulation. As we expected the release of Pc 4 became much faster in the pH 5.0 environment (30.7% Pc 4 released in 24 h). To investigate the effect of redox potential sensitiveness of TRN on its payload release, TRN was first incubated in pH 5.0 medium for 2 h and then transferred to pH 7.2 medium supplemented with 10 mM GSH to mimic the process of TRN escaping from lysosome to cytosol. Figure S3B revealed that the addition of GSH significantly accelerated the drug releasing process.

Head and neck squamous cell carcinoma (HNSCC) was selected to explore the PDT efficacy of TRN because most HNSCC cases are localized [21]. In addition, the treatment for HNSCC should not compromise the function and cosmetic appearance of corresponding tissues. All these make PDT, which causing minimal scar and loss of function of treated sites, a better alternative for surgery to treat HNSCC. Sigma-2 receptor is overexpressed in many cancers, including skin cancer, lung cancer, and breast cancer, and has been extensively explored as a target for tumor specific drug delivery [22–26]. However, thus far, no research investigated the expression of sigma-2 receptor in head and neck cancer. The expression of sigma-2 receptor in HNSCC was evaluated with immunohistochemistry in a human tissue array. High density of brown staining in the tumor tissue (Figure 3A) and little staining in the normal tissue (Figure 3B) indicated that sigma-2 receptor does overexpress in human head and neck tumor tissue, which makes it a valid target for tumor specific drug delivery. The quantitative analysis of the DAB-stained tissues revealed that human HNSCC expressed > 3-fold of sigma-2 receptor than normal tissues (Figure 3C). After that, we further confirmed that sigma-2 receptor is expressed in UMSCC22A head and neck cancer cells by Western immunoblotting (Figure 3D). Thus, UMSCC22A cell line was selected to validate our hypothesis in vitro.

To endow head and neck tumor targeting effect for TRN, a sigma-2 receptor targeting ligand, 4-methoxybenzoic acid (MBA) [26], was grafted onto PDA-PEG-PNIPMA with the help of 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) and N-hydroxysuccinimide (NHS) (Figure 1A). MBA-Pc 4-TRNs with different MBA densities were fabricated by adjusting the molar ratio of MBA-PDA-PEG-PNIPMA to PDA-PEG-PNIPMA. To investigate the targeting effect of MBA on the cellular uptake of Pc 4 loaded TRN, confocal microscopy was employed. Red fluorescence signal (Pc 4) was observed among all treatments (Figure 3E). Compared with free Pc 4, non-targeted TRN showed similar capacity in carrying Pc 4 into UMSCC22A cells during 20 h of incubation. As we expected, the functionalization of TRN with MBA significantly enhanced its cellular uptake. To further quantify the intracellular Pc 4 amount after 20 h of incubation, the cells were harvested and lysed to measure the intracellular Pc 4 amount. MBA-TRN with 16% ligand density achieved about 1.8-fold of Pc 4 uptake compared with that of non-targeted TRN (Figure 3F). The higher the MBA density, the better its cellular uptake, which suggest that the modification of MBA did facilitate the sigma-2 receptor mediated endocytosis for Pc 4 loaded TRN.

Since TRN was taken up by cells *via* endocytosis, we assessed the sub-cellular localization of MBA-Pc 4-TRN in UMSCC22A cells after it entered cells by confocal microscopy. Cells were incubated with 200 nM Pc 4-TRN (blue) for 2, 3, and 20 h and subsequently co-loaded with LysoTracker Green (LTG, green) and tetramethylrhodamine methylester (TMRM, red) to label endosomes/lysosomes and mitochondria, respectively. After 2 h, the presence of small round green/light cyan spheres representing lysosomes indicate that Pc 4-TRN began to enter the lysosomes but very few co-localization of Pc 4-TRN with mitochondria (red) was observed (Figure 4A). After 3 h, much more cyan spheres appeared, suggesting more TRN entered lysosomes; furthermore, the color of mitochondria turned from red to magenta, indicating strong co-localization between mitochondria and Pc 4 (Figure 4B). After 21 h, blue fluorescence became diffused, showing the expanding of TRN resulted in the release of Pc 4 (Figure 4C). Moreover, mitochondria exhibited stronger magenta fluorescence, indicating more Pc 4 transferred to mitochondria. It is also worth mentioning that we also observed some enlarged lysosome (cyan dots in Figure 4B and 4C), suggesting the expanding of TRN inside the lysosome during its intracellular traffic. To further quantitatively monitor the intracellular trafficking of TRN, images ($n > 15$) taken at different time points were analyzed by ImageJ to calculate the Pearson's co-localization coefficient for lysosome and Pc 4 (Pc 4/L), as well as Pc 4 and mitochondria (Pc 4/M). Figure 4D shows that more TRNs entered lysosomes after 2 h of incubation than that of 1 h ($p < 0.05$). The stronger co-localization of Pc 4 and mitochondria occurred after 3 h of incubation ($p < 0.01$). Since more TRN entered cancer cells (Figure S4) after 20 h of incubation, the co-localization of Pc 4/L further increased; as a consequence, more Pc 4 partitioned to mitochondria after it was freed from TRN.

To validate whether the enhanced uptake of Pc 4 and effective mitochondria targeting could be translated into better PDT efficacy in cell killing, cell viability after PDT was assessed by propidium iodide (PI) fluorometry [16]. UMSCC22A cells were incubated with non-targeted and targeted Pc 4-TRN for 20 h prior to receiving PDT. Cells treated with the same dose of nanoparticle receiving no light were employed as control. Figure 4E showed that PDT of MBA-Pc 4-TRN killed almost all cancer cells 12 h post irradiation, while only 44.5% cells were killed in the non-targeted TRN treated group. Combining cellular uptake data from Figure 3E and 3F, we concluded that better cellular uptake of Pc 4 did translate into better PDT cell killing efficacy. Furthermore, no cytotoxicity appeared in either TRN groups without applying light irradiation, indicating MBA-Pc 4-TRN and nano-carrier itself were safe.

To evaluate the tumor specific targeting effect of MBA-Pc 4-TRN, head and neck cancer xenograft mice model was employed. Mice were administered with MBA-Pc 4-TRN through the tail vein injection. Fluorescence images were taken with an *in vivo* fluorescence imaging system. At 72 h post-injection, MBA-Pc 4-TRN signal mainly appeared in the regions of tumor and liver, some in the bladder but very little in other tissues (Figure 4F). The *ex vivo* images obtained from dissected organs at 96 h post Pc 4 injection revealed that the majority of MBA-Pc 4-TRN was still retained in the tumor, while liver showed much less fluorescence signal compared to that of 72 h time point (Figure 4G). As expected,

spleen, heart, lungs and kidney retained very little Pc 4, which is significantly different from other Pc 4 loaded carrier systems [27, 28].

4. Discussion

PDT causing minimal scar and loss of function of treated sites, has been proposed as an alternative for surgery to treat HNSCC. However, the clinical application of PDT has been hindered due to the poor water solubility and non-specific skin retention of PS, as well as low PDT efficacy. To address that, we designed a thermal, pH, and redox potential triple-responsive expansile nanogel system (TRN), which swells at a temperature higher than its transition temperature, acidic pH, and reducing environment. *In vivo* biodistribution experiment revealed that TRN could specifically target tumor tissue with the synergetic outcome of EPR effect and sigma-2 receptor targeting effect (Figure 4G). The immunohistochemistry analysis of HNSCC human tissue array found that human HNSCC expressed > 3-fold of sigma-2 receptor than normal tissues (Figure 3C). In addition, Western immunoblotting confirmed that sigma-2 receptor is expressed in UMSCC22A head and neck cancer cells (Figure 3D), which makes UMSCC22A cell line a valid model for the study of sigma-2 receptor targeted therapy. Based on the experimental observations, we proposed the following pathway for MBA-Pc 4-TRN: (i) MBA-Pc 4-TRN entered head and neck cancer cells by sigma-2 receptor mediated endocytosis with the help of sigma-2 receptor ligand, MBA (Figure 3E and F); (ii) After that, MBA-Pc 4-TRN was transferred to endosome and then lysosome, where it has low pH. Partial of Pc 4 could be released from TRN due to the acidic pH (Figure S3A); (iii) Due the proton sponge effect of pyridine segments in PDA and the expansile property of TRN at low pH (Figure 2D and 2H), TRN and freed Pc 4 could escape from lysosome and enter cytosol; (iv) Since cytosol has an elevated GSH concentration, which can trigger the dramatic size expansion of TRN (Figure 2C, 2G, 2I, 4B, and 4C) and induce the release of Pc 4 (Figure S3B); (v) Due to its hydrophobicity, Pc 4 spontaneously transferred to mitochondria (Figure 4C and 4D). Therefore, MBA-Pc 4-TRN exhibited enhanced cell killing effect after PDT (Figure 4E).

Due to the advantages of PDT mentioned above, several of PS nanocarrier systems have been developed for HNSCC treatment [27–32]. Among them, only a few were evaluated *in vivo*. Wang et al. showed that an integrin $\beta 1$ binding fibronectin-mimetic peptide increased the accumulation of Pc 4 loaded iron oxide nanoparticle in HNSCC tumor [29]. However, this strategy also caused significantly higher Pc 4 retention in lung, liver, kidney, and skin tissues. In order to shorten the waiting time between drug administration and PDT treatment, Cheng et al. attached Pc 4 to gold nanoparticles through N-Au bonds [28]. Although the waiting period was reduced from 2 days to 2 h, this gold-Pc 4 system still couldn't solve the high lung and kidney retention associated with Pc 4. Since EGFR is overexpressed in most HNSCC, Master et al. functionalized PCL-PEG micelles with a EGFR targeting peptide, GE11. With the help of GE11, the targeted micelles exhibited higher cellular uptake and cell killing effect than the non-targeted ones [30]. Though, the high skin Pc 4 retention issue remaining unaddressed. To tackle the non-specific retention of Pc 4, we integrated a triple-responsive nanogel system with a sigma-2 receptor targeting ligand, MBA. MBA functionalized Pc 4-TRN showed much better cellular uptake than Pc 4-TRN and killed more head and neck cancer cells after PDT. Because the existence of a dense PEG layer on

the surface of TRN, which prevents non-specific bindings, only negligible amount of Pc 4 retained in lung, kidney, and other tissues. Therefore, MBA-Pc 4-TRN achieved HNSCC tumor specific targeting effect.

5. Conclusions

In summary, a thermal, pH, and redox potential triple-responsive expansile nanogel system (TRN) has been developed. The transition temperature of TRN could be tuned from 30.5 °C to 47 °C by adjusting its crosslinking density. Due to the synergistic effect of its redox potential and thermal responsiveness, TRN could expand from 108 nm to over 1200 nm within 2 h in a reducing environment at body temperature, achieving more than 1000-fold size enlargement (in volume). Pc 4 loaded TRNs are stable (both size and retaining loaded drug) in a physiological condition, while quickly releasing Pc 4 at lysosomal pH and reducing cytosol environment attributed to its rapid swelling response upon the trigger of acidic pH, high temperature, and elevated GSH. MBA functionalized Pc 4-TRN could effectively enter UMSC22A cancer cells with the help of sigma-2 receptor and transfer Pc 4 to its target, mitochondria. Consequently, PDT of MBA-Pc 4-TRN showed significant higher toxicity than its non-targeted counterpart and killed almost all cancer cells. Furthermore, *in vivo* biodistribution study proved that MBA-Pc 4-TRN could effectively target head and neck tumor tissue and be retained there for 4 days. Based on the unique responsiveness and promising *in vitro* and *in vivo* results from TRN, further studies will focus on the mechanisms for TRN escaping from lysosome and employing the system for *in vivo* tumor growth inhibition effect for head and neck cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A: Supporting Information

¹H NMR and GPC spectra of PDA-PEG-PNIPMA polymer; stability of TRN at 37 °C monitored by dynamic light scattering; Pc 4 release kinetics of TRN at different conditions; and cellular uptake of Pc 4 after 4 and 20 h of culture. Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/>.

Figure 1.

(A) Schematic illustration of the synthesis of MBA-PDA-PEG-PNiPMA polymer and the fabrication of MBA-Pc 4-TRN nanogels, (B) the relationship between TRN crosslinking density and its transition temperature. The transition temperatures were determined by measuring the change of transmittance for TRN PBS suspension at the nanogel concentration of 0.16 mg/ml during the increase of temperature.

Figure 2.

The z-average size of TRN in response to the change of temperature (A, B), the addition of 10 mM DTT (C), and the change of pH from 7.4 to 5.0 (D) acquired by DLS. All the size measurements were carried out at 37 °C unless otherwise specified. TEM images of control TRN at room temperature (E), heated at 42 °C (F), treated with 10 mM DTT for 2 h at 37 °C (G), and incubated in pH 5.0 buffer (H). Images were taken with a Hitachi H8000 TEM. Scale bars are 200 nm in (E) and (F), and 500 nm in (G) and (H). The size distribution of TRN in response to the addition of 10 mM DTT over time (I).

Figure 3.

The expression of sigma-2 receptor in head and neck cancer and its effect on the cellular uptake of MBA-Pc 4-TRN. The expression of sigma-2 receptor in normal tissue (A) and human head and neck cancer tissue (B). Representative images shown. Sigma-2 receptor positive tissue areas were detected with immunohistochemistry and quantified as percent of total tissue and expressed as fold of normal tissue (mean ± SE, n >30, # p < 0.001) (C); The expression of sigma-2 receptor in UMSCC22A cell line detected by Western immunoblotting (D); Confocal images of UMSCC22A cells treated with Pc 4 or Pc 4 loaded TRN (E). Cells were cultured with Pc 4 or Pc 4 loaded TRN (red) for 20 h. Cell nuclei were stained with DAPI (blue). Images from left to right were taken from DAPI, Pc 4, transmittance, and the overlay of the previous three channels; (F) Intracellular amount of Pc 4 for cells treated with Pc 4-TRNs containing different MBA densities (mean ± SD, n = 3).

Figure 4.

Subcellular co-localization of Pc 4 loaded TRN (red) with lysosome and mitochondria recorded at 2 h (A), 3 h (B), and 21 h (C) by confocal microscopy. LysoTracker Green (LTG, green) and tetramethylrhodamine methylester (TMRM, red) were used to label endosomes/lysosomes and mitochondria, respectively. Scale bar in (C) is 10 μ m; (D) The trend for the Pc 4 co-localizing with lysosome (Pc4/L) and mitochondria (Pc 4/M) (mean $\pm$ SD, $n > 15$; * $p < 0.05$; # $p < 0.01$); (E) The PDT efficacy in killing UMSCC22A cells 12 h after irradiation (mean $\pm$ SD, $n = 3$; # $p < 0.01$); (F, G) Biodistribution of MBA-Pc 4-TRN *in vivo*. Xenografts were created with UMSCC22A cells in nude mice. MBA-Pc 4-TRN was administered *via* tail vein. Mice were imaged before (F, left) and 72 h after drug administration (F, right). At 96 h, organs and tumor were dissected from the mouse treated with MBA-Pc 4-TRN and imaged *ex vivo* (G). Representative images shown.