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Stability enhanced polyelectrolyte-coated gold nanorod-photosensitizer complexes for high/low power density photodynamic therapy

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Abstract

Photodynamic therapy (PDT) is a promising treatment modality for cancer and other malignant diseases, however safety and efficacy improvements are required before it reaches its full potential and wider clinical use. Herein, we investigated a highly efficient and safe photodynamic therapy procedure by developing a high/low power density photodynamic therapy mode (high/low PDT mode) using methoxypoly(ethylene glycol) thiol (mPEG-SH) modified gold nanorod (GNR)-AlPcS4 photosensitizer complexes. mPEG-SH conjugated to the surface of simple polyelectrolyte-coated GNRs was verified using Fourier transform infrared spectroscopy; this improved stability, reduced cytotoxicity, and increased the encapsulation and loading efficiency of the nanoparticle dispersions. The GNR-photosensitizer complexes were exposed to the high/low PDT mode (high light dose = 80 mW/cm² for 0.5 min; low light dose = 25 mW/cm² for 1.5 min), and a high PDT efficacy leads to approximately 90% tumor cell killing. Due to synergistic plasmonic photothermal properties of the complexes, the high/low PDT mode demonstrated improved efficacy over using single wavelength continuous laser irradiation. Additionally, no significant loss in viability was observed in cells exposed to free AlPcS4 photosensitizer under the same irradiation conditions. Consequently, free AlPcS4 released from GNRs prior to cellular entry did not contribute to cytotoxicity of normal cells or impose limitations on the use of the high power density laser. This high/low PDT mode may effectively lead to a safer and more efficient photodynamic therapy for superficial tumors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2014.04.105>.

Keywords

High/low power density; ALPcS4 photosensitizer; Gold nanorods; Photodynamic therapy (PDT); Near-infrared; Synergistic therapy

1. Introduction

Photodynamic therapy (PDT) is a medical intervention to remove unwanted biological agents that requires three essential components: light, photosensitizer, and oxygen [1,2]. Upon light irradiation at a specified wavelength and appropriate dose of light, the photosensitizer converts endogenous oxygen to singlet oxygen ($^1\text{O}_2$), leading to the death of cancer cells. Since light irradiation does not directly damage neighboring cells or those that have not been exposed to the photosensitizer, PDT can remarkably improve cell selectivity and has fewer side effects compared to conventional chemotherapeutic and radiotherapeutic methods.

With the development of nanotechnology, the delivery of photosensitizer by nanomaterials has received much attention in recent years. Nano-formulation allows passive targeting of tumors through “enhanced permeability and retention” (EPR) effect [3,4] to improve the treatment efficacy in PDT. In previous reports, gold nanorod (GNR)/photosensitizer complexes have been widely used for combined photodynamic therapy/photothermal therapy (PDT/ PTT) to obtain high therapeutic efficacy, because GNRs with strong and controlled surface plasmon absorption bands in the NIR region can serve as ultra-efficient energy quenchers and photothermal agents [5-7]. Gold nanorods with “confined” thermal effects is widely used for photothermally activated drug and gene delivery, based on the plasmonic photothermal effect [8-10]. The measurement and control of light dose in vivo have also been extensively studied to reduce the side effects of PDT and realize the accurate treatment [11-13]. Proper selection of the photosensitizer and the gold nanomaterials allows single continuous wave laser induced PDT/PTT to reduce treatment time and eliminate the need for precise alignment of the two light beams [14,15]. However, photosensitizers that are electrostatically adsorbed onto the surface of GNRs will release in the body, resulting in uncontrollable damage to normal cells [5,6]. Thus accurate, ultra-highly efficient and safe photodynamic therapy combined with nanotechnology would be the focus of the future research in this field.

Herein, we describe a mode of high power density combined with low power density photodynamic therapy (high/low PDT; below the 200 mW/cm^2 ANSI Z136.1 laser safety limit) induced by a single continuous wave laser, using stability enhanced simple polyelectrolyte coated GNR-photosensitizer complexes. mPEG-SH conjugated to the surface of the polyelectrolyte coated GNR-photosensitizer complexes was designed to evaluate stability, cytotoxicity, encapsulation and loading efficiencies; all of these are desired attributes for PDT-induced ablation of cancer cells. As conceptually illustrated in the Fig. 1 schematic, compared to only high power density PDT and only low power density PDT, high (short duration)/low (long duration) power density PDT mode was designed to realize a highly efficient and safe photodynamic therapy when cells were exposed to GNR-photosensitizer complexes or free photosensitizer, eliminate the cytotoxicity to normal cells

by the free photosensitizer released from GNRs prior to cellular entry and reduce the use of high power density laser to decrease the treatment risk. Additionally, single continuous wave laser was used to simultaneously apply the plasmonic photothermal release of photosensitizer and photodynamic therapy.

2. Materials and methods

2.1. Materials

Sodium borohydride (NaBH_4), cetyltrimethylammonium bromide (CTAB), silver nitrate (AgNO_3) and ascorbic acid (AA), hydrogen tetrachloroaurate(III) trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) were purchased from Sinopharm Chemical Reagent Co., Ltd (Beijing, China). Poly(sodium 4-styrenesulfonate) (PSS, $M_w \approx 70,000$ Da), branched poly(ethyleneimine) (BPEI, $M_w \approx 25,000$ Da), FITC labeled phalloidine were purchased from Sigma–Aldrich. Al(III) phthalocyanine chloride tetrasulfonic acid (AlPcS4) was purchased from Frontier Scientific, Inc. Methoxypoly(ethylene glycol) thiol (mPEG-SH, $M_w \approx 5000$ Da) was obtained from Xiamen Sinopeg Biotech Co., Ltd. 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and nuclear dyes Hoechst 33342 were obtained from Ningbo Hangjing biotechnology Co. Ltd. (Ningbo, China). Fetal bovine serum (FBS), Dulbecco's modified eagle's medium (DMEM), penicillin-streptomycin solution and trypsin-EDTA solution were purchased from Gibco (Grand Island, USA). Milli-Q water ($18\text{M}\Omega\cdot\text{cm}$) was used for all solution preparations. All chemicals were analytical grades as received.

2.2. Synthesis of gold nanorods

GNRs were synthesized by a seed-mediated method as described by Murphy et al. [16] The synthesis volume was magnified 15 times, briefly: the Au seeds were prepared by mixing 0.25 mL HAuCl_4 solution (0.01 M) with 7.50 mL of 0.10 M CTAB solution. While the solutions were gently mixed, 0.60 mL of 0.01 M NaBH_4 was added and stirred rapidly for 2 min. At the same time, 71.25 mL of 0.10 M CTAB, 3.00 mL of 0.01 M HAuCl_4 and 0.45 mL of 0.01 M AgNO_3 solutions were added sequentially, followed by gentle stirring. The solution at this stage appeared bright brown-yellow in color. Next, 0.48 mL of 0.10 M AA was added to the brown-yellow solution and it became colorless. Finally, 0.15 mL of Au seeds were added and reacted for 3 h to obtain the GNR-CTAB.

2.3. Synthesis of GNR-PSS-PEI-mPEG-SH nanocarrier

The preparation of polyelectrolyte-coated GNRs was obtained *via* a layer-by-layer approach [17-19]. The multilayer polyelectrolyte-coated GNRs were synthesized by sequentially electrostatically coating negatively charged poly(sodium 4-styrenesulfonate) (PSS) and positively charged branched poly(ethyleneimine) (BPEI) onto the positively charged GNR-CTAB. During the PSS coating, 20 mL of as-synthesized GNR-CTAB was centrifuged twice ($14,000\text{ g}$) to remove excess CTAB, and the precipitate was dispersed in 20 mL of 2 mg/mL PSS aqueous solution (containing 6 mM NaCl). The solution was stirred magnetically for 4 h, followed by a $14,000\text{ g}$ centrifugation for 15 min to obtain GNR-PSS. The precipitate of GNR-PSS was redispersed in 20 mL of 2 mg/mL BPEI aqueous solution (containing 6 mM NaCl) and stirred magnetically for an additional 4 h. Next, the mixture was centrifuged at $14,000\text{ g}$ for 10 min to obtain GNR-PSS-PEI and then resuspended in 20

mL of deionized water. In order to improve the stability of polyelectrolyte-coated GNRs, 2 mL of mPEG-SH (1 mM) was added to 20 mL of GNR-PSS-PEI solution, and the mixture was stirred at room temperature for 24 h to form Au–S bonds. Finally, the solution was centrifuged at 14,000 g for 15 min, decanted, and redispersed in deionized water to obtain the GNR-PSS-PEI-mPEG-SH nanocarrier. The formation of Au–S bond was confirmed by Fourier transform infrared spectroscopy (FT-IR) (Nicolet 6700, Thermo scientific).

2.4. Synthesis of mPEG-SH modified GNR-AIPcS4 complexes

The mPEG-SH modified GNR-AIPcS4 complexes were prepared by mixing the positively charged GNR-PSS-PEI-mPEG-SH with the negatively charged photosensitizer Al(III) phthalocyanine chloride tetrasulfonic acid (AIPcS4), which is a second-generation photosensitizer that possesses optical properties well-suited for PDT. First, an AIPcS4 aqueous solution (10 μ L, 2.8 mM) was added to 4 mL of 0.5 nM GNR-PSS-PEI-mPEG-SH, and the mixed solution was stirred at room temperature for 4 h to form a charged complex. Centrifugal filters (100,000 Mw, Amicon Ultra, Millipore Ireland, Ltd.) were used to purify the GNR-AIPcS4 complexes modified by mPEG-SH. The UV–Vis absorption spectrum of unadsorbed free AIPcS4 was measured using UV–Vis spectrophotometer (T10CS, Purkinje General Co., Ltd.) to calculate the average number of AIPcS4 attached per GNR. The extinction coefficient of GNR-CTAB was about $4.28 \times 10^9 \text{ M}^{-1} \text{ cm}^{-1}$ at 760 nm [6,20]. AIPcS4 is known to have an extinction coefficient of $1.7 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ at 675 nm [6,21]. The molar concentrations of GNRs (0.5 nM GNR-CTAB, $\text{OD}_{760\text{nm}} = 2.14$) and AIPcS4 were determined by of UV–Vis absorption spectra.

2.5. Plasmonic photothermal release of AIPcS4 from nanocarrier

AIPcS4 releasing experiments from GNR-PSS-PEI-mPEG-SH nanocarriers were investigated in $1 \times \text{PBS}$ with and without light irradiation. A dialysis membrane (Mw = 3 kDa) was filled with a solution containing mPEG-SH modified GNR-AIPcS4 complexes (4 mL, 1.5 nM GNRs, 14 μ M AIPcS4 equiv.), immersed in 33 mL of PBS solution, and gently stirred at 37 °C for 48 h. The solution outside the dialysis membrane was collected after each test, and the amount of AIPcS4 released was measured by a UV–Vis spectrometer at 675 nm. The PBS solution outside the dialysis membrane was changed for each new test. A similar procedure was used in the NIR laser controlled AIPcS4 releasing experiment, but the solution of mPEG-SH modified GNR-AIPcS4 complexes was irradiated with a NIR laser (wavelength, 671 nm; beam diameter, 1 cm; power, 500 mW) for 5 min at scheduled time intervals.

2.6. Cell culture

MCF-7 human breast cancer cell line was obtained from Ningbo No. 2 Hospital. The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified incubator at 37 °C in air with 5% CO_2 .

2.7. In vitro cytotoxicity of GNR nanocarrier

In vitro cytotoxicity of GNR nanocarrier was measured by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. Typically, 100 μ L of MCF-7

cells were seeded in each well of 96-well plates at a density of 1×10^4 cells/ well and incubated for 24 h. Thereafter, the cells were incubated with 100 mL of fresh DMEM with various concentrations of GNR-CTAB, GNR-PSS-PEI, or GNR-PSS-PEI-mPEG-SH containing 10% FBS for another 24 h. Subsequently, 10 μ L of MTT (5.0 mg/mL in PBS) was added into each well. After 4 h of incubation, the entire medium with MTT was removed and 100 mL of DMSO was added to each well to dissolve the formed formazan crystals. Finally, the absorbance of each well was measured at 550 nm using a Microplate Reader (iMark 168-1130, Bio-rad) to calculate the cell viability. For the untreated control group, the same volume of fresh culture medium without nanocarrier was added to the plate. Cell viability of each treatment was compared to the untreated control group and expressed as a percentage.

2.8. Confocal laser scanning microscopy

MCF-7 cells (1 mL, 1×10^5 , determined by cell counting board) were seeded into 35 mm culture dishes and incubated for 24 h. The growth media were then replaced by fresh DMEM, free AlPcS4 (14 μ M), or mPEG-SH modified GNR-AlPcS4 complexes (1 nM, 14 μ M AlPcS4 equiv.). After incubation for 4 h, cells were washed three times with PBS, fixed with 4% formaldehyde for 30 min, washed 3 times with PBS, incubated with 0.2% Triton X-100 for 10 min, and rinsed with PBS again. Next, a 1% BSA solution was added for 25 min to block nonspecific binding sites. The cells were then stained with 200 μ L (50 μ g/mL) of FITC-phalloidine for 1 h at 37 °C and washed 3 times with PBS. Finally, the cells were stained with 1 mL of Hoechst 33342 (2 μ g/mL) for 15 min and washed 3 times with PBS. All samples were then imaged with a Leica TCS SP5 confocal microscope (Leica Microsystems, Germany). Excitation and emission wavelengths (nm) used for Hoechst 33342, FITC-phalloidine, and AlPcS4 were 405 nm/420–480 nm, 488 nm/500–540 nm, and 633 nm/636–721 nm, respectively.

2.9. In vitro laser-induced photodynamic therapy

MCF-7 cells were plated in 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h. The existing culture medium was then replaced with DMEM with 10% FBS containing either 100 μ L of free AlPcS4 (7 μ M) or mPEG-SH modified GNR-AlPcS4 complexes (0.5 nM, 7 μ M AlPcS4 equiv.) for another 4 h. For the untreated control group, the same volume of fresh culture medium (without photosensitizer) was added to the plate. After 4 h of incubation, the cells were washed and fresh cell culture medium was added. The PDT-treated groups were immediately irradiated with 671 nm continuous wavelength (CW) laser beam at different power densities and irradiation times. The cells were incubated for another 24 h, and the cell viability was measured using MTT assay. The toxicity of dark control treated by free AlPcS4 or mPEG-SH modified GNR-AlPcS4 complexes were kept identical to the experimental group except for there was an absence of irradiation. The power densities (mW/cm^2) used in photodynamic therapy was determined by the ratio of fiber emitted power (instrument settings) and beam area in the sample plane.

3. Results and discussion

3.1. Synthesis and characterization of mPEG-SH modified GNR-AIPcS4 complexes

GNR-CTAB was prepared by a modified seed-mediated method previously described [16]. GNR-PSS-PEI was obtained *via* a well-developed layer-by-layer assembly approach [17-19]. The toxic CTAB on the surface of GNR was wrapped by the PSS and PEI to reduce cytotoxicity. In order to improve the stability, mPEG-SH was conjugated on the surface of GNRs through the formation of Au-S bonds. The positively charged amino group of the BPEI was introduced to form a charged complex with the negatively charged AIPcS4 photosensitizer (Fig. 2).

The UV-Vis-NIR absorption spectra (normalized at 511 nm, Fig. 3a) showed that the longitudinally localized surface plasmon resonance (LSPR) peak of GNR-CTAB was 760 nm (the aspect ratio of nanorods, $R = 3.0 \pm 0.4$). The LSPR peaks of GNRs with sequentially different surface coatings decreased and showed a little red-shift and widening of the peak shape after repeated centrifugation and redispersed in several modification steps. The size distribution and morphology of GNRs with sequentially different surface coatings were characterized by Dynamic Light Scattering (DLS) (Nano ZS, Malvern) and Transmission Electronic Microscopy (TEM) (JEOL2100 HR, JEOL) (Fig. S1). In this study, the average size of DLS results only represented the change of the size of GNRs with sequentially different surface coatings, which could not characterize the hydrodynamic size of GNRs (anisotropic nanoparticle). The application of these previously described modification steps resulted in the size of GNR nanoparticles becoming a little larger, shown in Fig. 3c and Fig. S1, resulting in a little red-shift and widening of the LSPR peak shape. After a large number of AIPcS4 photosensitizer adsorption, a faint layer could be seen around the dark GNRs. The rough estimate of the thickness of polyelectrolyte layer on the surface of GNRs was nearly 2.3 nm (Fig. 3b). Direct visualization of polymer coating of the GNRs can be achieved by high-magnification TEM measurements [17]. From the TEM images, the prepared GNRs had a dogbone shape using the seed-mediated method as described by Murphy et al. [16] In this study, the GNRs were only used as the nanocarrier and the media with plasmonic photothermal effect. Zeta-potential results showed that the GNR-CTAB and the GNR-PSS-PEI were positively charged, and the GNR-PSS was negatively charged. The conjugation of mPEG-SH and the adsorption of AIPcS4, decreased the positive charge of GNRs.

Dispersion stability of the nanoparticles is very important for biological applications. Though the method of polyelectrolyte modified is simple and widely used [17-19], the dispersion stability was not ideal in PBS or 10% FBS solution as shown in Section 3.3. Thus, improving the stability of polyelectrolyte coated GNRs is critical for biological applications. In order to improve stability, mPEG-SH was used to modify the surface of polyelectrolyte coated-GNRs through the formation of Au-S bonds. The formation of Au-S bonds was confirmed by FT-IR in the region of $150\text{--}450\text{ cm}^{-1}$ (Fig. S2). The IR spectra of GNR-CTAB + mPEG-SH confirmed the experimental Au-S stretching vibration around 165 cm^{-1} and 220 cm^{-1} , compared to the GNR-CTAB. In different crystal planes or modifications, the IR peak of Au-S stretching vibrations were not the same, such as 278.5

cm^{-1} [22] (Au NPs modified by $[\text{SCH}_2\text{CO}_2]^{2-}$), 220 cm^{-1} (methanethiol on Au(111)) and 235 cm^{-1} (dimethyl disulfide on Au(111)) [23]. The Au–S stretching vibration peak of GNR-PSS-PEI + mPEG-SH was close to 165 cm^{-1} and 230 cm^{-1} , and the relative intensity of 230 cm^{-1} was lower than that of GNR-CTAB + mPEG-SH (220 cm^{-1}). The results showed an Au–S bond was formed between GNR-PSS-PEI + mPEG-SH. However, due to the self-assembly of polyelectrolyte on GNR surface, the contact probability of –SH and gold surface decreased; this resulted in the formation ability of Au–S bond reduced and the relative intensity decreased. The possible mechanism for forming an Au–S bond on the surface of GNR-PSS-PEI was due to mutual, electrostatic repulsion between the polyelectrolyte itself. The poly-electrolyte layer could not wrap tightly onto GNR-CTAB surface, so that the mPEG-SH could contact with the gold surface to form Au–S bond through the gap.

3.2. Encapsulation and loading efficiency of mPEG-SH modified GNR-AIPcS4 complexes

Different concentrations ($2.1\text{ }\mu\text{M}$, $4.6\text{ }\mu\text{M}$, and $7\text{ }\mu\text{M}$) of AIPcS4 solution were added to 0.5 nm GNR-PSS-PEI and GNR-PSS-PEI-mPEG-SH nanocarriers and mixed for 4 h at room temperature, and the mixture was then centrifuged at $14,000\text{ g}$ for 20 min to obtain the unadsorbed AIPcS4 solution. At the same time, the absorbance at 675 nm of the unadsorbed AIPcS4 solution was determined by the UV–Vis spectrometer. The AIPcS4 encapsulation efficiency was calculated through the formula: encapsulation efficiency (%) = (total AIPcS4 – unadsorbed AIPcS4) $\times$ 100%/total AIPcS4. The encapsulation efficiency of GNR-PSS-PEI-mPEG-SH was higher than GNR-PSS-PEI, and was nearly 100% (Fig. S3). The adsorption rate of GNR-PSS-PEI-mPEG-SH was quick, as demonstrated by more than 90% adsorption in less than 5 min. Conjugation of the linear mPEG-SH may have contributed to the high adsorption rate and efficiency of GNR-PSS-PEI-mPEG-SH. The conjugation of linear mPEG-SH may have enhanced the spatial stability of the nanoparticles. Additionally, mPEG-SH may act as a “brush” to help the small AIPcS4 molecules to adsorb onto BPEI and reduce the chances of escape from the surface of GNRs. The zeta potential was reduced after the AIPcS4 was adsorbed (Fig. S4). The mPEG-SH modified GNR-AIPcS4 complexes became aggregated if more AIPcS4 ($14\text{ }\mu\text{M}$) was adsorbed. AIPcS4 ($7\text{ }\mu\text{M}$) adsorbed on 0.5 nm GNR-PSS-PEI-mPEG-SH was stable as shown in Section 3.3. The molar ratio of bound AIPcS4 per GNR (loading efficiency) was about 14000:1.

3.3. Stability of mPEG-SH modified GNR-AIPcS4 complexes

The surface chemistry of nanoparticles including GNRs played an essential role in improving their hydrophilicity, colloidal stability, and biocompatibility which are very important for biological applications [24–26]. Thus, the dispersion stability of mPEG-SH modified GNR-AIPcS4 complexes in deionized water, PBS and 10% FBS was tested prior to the *in vitro* cellular studies. For comparison, GNR-PSS-PEI, GNR-PSS-PEI-AIPcS4, GNR-PSS-PEI-mPEG-SH and GNR-PSS-PEI-mPEG-SH-AIPcS4 were dispersed in deionized water, PBS and 10% FBS. In PBS solution, GNR-PSS-PEI and GNR-PSS-PEI-AIPcS4 were not stable. The absorption spectra of each of them changed significantly over time (Fig. S5a and b), becoming fully aggregated after 7 days (Fig. S8a and b). Following mPEG-SH conjugation, the stabilities of both GNR-PSS-PEI-mPEG-SH and GNR-PSS-PEI-mPEG-SH-AIPcS4 were greatly improved. Fig. S5c and d show that the absorption spectra

did not change a lot after 7 days (the absorption of LSPR reduced about 20%). Though the PEGs on the surface of GNRs prevented nanorod aggregation in PBS, similarly modified GNR-PSS-PEI-mPEG-SH and GNR-PSS-PEI-mPEG-SH-AIPcS4 were not completely stable in PBS. The average sizes of these latter two increased near 100 nm after 7 days (Fig. S8c and d). The reason was that PEGs could only partially conjugate on the surface of GNRs because of the previously applied polyelectrolyte coat. Thus, PEGs on the surface of GNRs were not enough to maintain complete stability.

In 10% FBS, Fig. S6 showed that the absorption spectra of these four types of nanoparticles reduced a little. The hydrodynamic particle size increased nearly 50 nm due to the negatively charged serum proteins adsorbed, except in the case of GNR-PSS-PEI (Fig. S8). The protection of PEGs and the reduction of zeta potential after AIPcS4 adsorption improved the stability of the four types of nanoparticle complexes in 10% FBS solution. These four types of nanoparticles were all well-dispersed in deionized water, and the UV-Vis absorption spectra and average size were not changed (Fig. S7 and Fig. S8).

3.4. Plasmonic photothermal release of AIPcS4 from nanocarrier

The fluorescence emission and singlet oxygen generation (SOG) are controlled by the distance between GNRs and photosensitizers. When photosensitizer is located near the surface of GNRs, the laser energy transferring from photosensitizer to GNRs is effective, and photosensitizer may become non-fluorescent and non-phototoxic [6,27]. Thus, in our current study, mPEG-SH modified GNR-AIPcS4 complexes were non-fluorescent and non-phototoxic while in the circulatory system before the AIPcS4 release, because the distance between AIPcS4 and GNR of mPEG-SH modified GNR-AIPcS4 complexes was about 2.3 nm (Fig. 3b). Though low phototoxicity in the circulatory system improves biological safety, AIPcS4 releasing performance from GNRs is an important condition for its PDT efficacy. AIPcS4 releasing experiments from mPEG-SH modified GNR-AIPcS4 complexes with and without NIR laser irradiation in PBS solution were investigated. Fig. 4 indicated that AIPcS4 released nearly 90% with NIR laser irradiation (wavelength, 671 nm; beam diameter, 1 cm; power, 500 mW) for 5 min at scheduled time intervals after 48 h. This was much higher than that without NIR laser irradiation in PBS solution, due to the plasmonic photothermal effect of GNRs [8,9] and heat generation and temperature increase during light illumination [6,28]. In fact, owing to the limit of power density and treatment time of photodynamic therapy, AIPcS4 could only partially release from GNRs.

3.5. Cytotoxicity of GNR nanocarrier in MCF-7 cells

The surface chemistry of GNRs strongly influences their cell toxicity [29]. Thus, *in vitro* cytotoxicity to MCF-7 cells for different concentrations of GNR-CTAB, GNR-PSS-PEI and GNR-PSS-PEI-mPEG-SH was each determined by an MTT assay. The cytotoxicity to MCF-7 cells increased with increasing concentrations of GNR-CTAB, GNR-PSS-PEI and GNR-PSS-PEI-mPEG-SH (Fig. 5). The cytotoxicity of CTAB on the surface of GNR was reduced after wrapping the PSS and PEI. The cytotoxicity of GNR-PSS-PEI-mPEG-SH was lower than its two counterparts at the same concentration because of the improvement of biocompatibility. MCF-7 cells treated with GNR-PSS-PEI-mPEG-SH (0.5 nM) did not

exhibit any significant viability losses (the cell viability was near 90%), so this dose of nanoparticles was used in subsequent experiments.

3.6. Intracellular localization of AIPcS4

Because the action radius of cytotoxic singlet oxygen is less than 20 nm, the cellular uptake of photosensitizer is of great significance when using photodynamic therapy in cancer cells. This suggests that any photosensitizer accumulating in the extracellular space will not function as an effective PDT, even if they are accumulated within tumor tissue [6,30,31]. To investigate the behavior of intracellular free AIPcS4 and mPEG-SH modified GNR-AIPcS4 complexes, cellular internalization of the photosensitizer was visualized using confocal fluorescence microscopy (ex = 633, em = 636–721 nm) to evaluate the degree of photosensitizer uptake into the cancer cells and intracellular localization of AIPcS4. Even without considering the fluorescence quenching of AIPcS4 by GNRs, it is clear that the red fluorescence intensity inside MCF-7 cells treated with mPEG-SH modified-GNR-AIPcS4 complexes was greater than that inside MCF-7 cells treated with free AIPcS4 (Fig. 6 and S9). The uptake enhancement of photosensitizer into cancer cells using nanoparticles is extensively validated by the confocal laser scanning microscopy or flow cytometer [6,14,15]. More importantly, as shown in Fig. 6 and S9 about the intracellular localization of AIPcS4, free AIPcS4 was more likely to distribute throughout the whole cell (both in the cytoplasm and nucleus) evenly (low fluorescence). For mPEG-SH modified GNR-AIPcS4 complexes, red fluorescence centralized in the outer edge region of the cell. Due to the endocytic entry pathway of GNRs, most of the photosensitizer signal localized in high concentrations within cytosolic vesicles. A recent study using TEM had demonstrated that GNRs were easily taken up by cells *via* the endocytic pathway, gathering in the cytosolic vesicles such as the endosomes and lysosomes [6]. Cellular uptake of photosensitizer through mPEG-SH modified GNR-AIPcS4 complexes was expected to be generally greater and more localized than that of free AIPcS4.

3.7. High/low PDT mode

Typically, PDT studies using nanomaterials focus solely on the dose of light irradiation and type of photosensitizer. In this study, a novel approach was undertaken – high power density combined with low power density photodynamic therapy mode (high/low PDT) was investigated for the first time. The intracellular efficacy of this high/low PDT mode was measured using the MTT assay in MCF-7 cancer cells. The experiments only studied the PDT efficacy produced by the intracellular photosensitizer, so the cell culture medium containing mPEG-SH modified GNR-AIPcS4 complexes (7 μ M AIPcS4 equiv.) or free AIPcS4 (7 μ M) was replaced by the fresh culture medium after 4 h of incubation. To simplify the therapeutic system, a single continuous wave laser (671 nm) was used to simultaneously achieve controlled release and PDT treatment. However, the laser (671 nm) used to excite AIPcS4 was not the ideally suited wavelength to release AIPcS4 from GNRs (the LSPR peak of mPEG-SH modified GNR-AIPcS4 complexes was about 810 nm). The extinction coefficient of mPEG-SH modified GNR-AIPcS4 complexes at 671 nm was about 60% of 810 nm. Though the LSPR could be tuned by adjusting the aspect ratio of GNRs, the lower LSPR peak had lower extinction coefficients [32–34]. Thus, tuning the LSPR peak to 671 nm could not have effectively increased the extinction coefficient of GNRs. In the future,

other nanocarriers could be used to improve the plasmonic photothermal release of AlPcS4, such as gold nanostars [15].

Provided that the light dose was large enough, both free AlPcS4 and mPEG-SH modified GNR-AlPcS4 complexes had a notable therapeutic effect (Fig. 7). Under the light dose of high power density (400 mW/cm^2 , 3 min) and low power density (200 mW/cm^2 , 5 min), the cell viability was less than 20% for free AlPcS4, AlPcS4+GNR-PSS-PEI-mPEG-SH (mixed directly in the MTT assay) and GNR-PSS-PEI-mPEG-SH-AlPcS4. There were no statistically significant differences for each other ($**p > 0.05$). The control of free AlPcS4+GNR-PSS-PEI-mPEG-SH was discussed in this experiment. The photothermal therapy efficacy was also studied with controls and GNR-PSS-PEI-mPEG-SH after irradiation by the high light dose of high power density (400 mW/cm^2 , 3 min) and low power density (200 mW/cm^2 , 5 min). The results showed that there was no photothermal therapy efficacy by these light doses and the therapy efficacy was all produced by the PDT.

The lower optical power density and shorter irradiation times were discussed (Fig. 8). The results showed that the cell viability of free AlPcS4 was increased to 42% (Fig. 8a) and 65% (Fig. 8b), when the laser dose (671 nm) was high = 80 mW/cm^2 , 2min + low = 25 mW/cm^2 , 4 min and high = 80 mW/cm^2 , 1.5 min + low = 25 mW/cm^2 , 2 min, respectively. The PDT efficacy of mPEG-SH modified GNR-AlPcS4 complexes was still very efficient (cell death was close to 90%). The laser irradiation had an important influence on the cell viability incubated with free AlPcS4 and GNR-AlPcS4 complexes. The therapeutic effect of GNR-PSS-PEI-mPEG-SH-AlPcS4 and free AlPcS4 + GNR-PSS-PEI-mPEG-SH control was compared in Fig. 8(a) and (b). In the case of high PDT 1.5 min and low PDT 2 min, GNR-PSS-PEI-mPEG-SH-AlPcS4 showed higher therapeutic effect than the mixture of free AlPcS4 and GNR-PSS-PEI-mPEG-SH at the same concentration ($*p < 0.05$) with the decrease of the light dose, which could be attributed to the enhanced cellular uptake efficiency of AlPcS4 after conjugation with GNR-PSS-PEI-mPEG-SH.

When irradiation time was further reduced to high = 80 mW/cm^2 , 0.5 min and low = 25 mW/cm^2 , 1.5 min, there was a surprising finding that the high/low PDT mode obtained ultra-high PDT efficacy (about 90%) by GNR-PSS-PEI-mPEG-SH-AlPcS4 and no significant viability loss by free AlPcS4 ($*p < 0.01$ and $**p < 0.01$), shown in Fig. 9. The high power density light (80 mW/cm^2) was only used 0.5 min. In the case of High = 80 mW/cm^2 , 1 min + low = 25 mW/cm^2 , 2 min, there was significant viability loss by free AlPcS4 ($*p < 0.05$), shown in Fig. S10. When the high PDT time and low PDT time were reduced to 20 s and 60 s, respectively, the cell viability of high/low PDT mode increased to 40% (Fig. 10). Thus, the choice of laser power density and irradiation time was the key to the high/low PDT mode, which was changed by different treatment systems (such as different nano-carriers and different doses of photosensitizer etc.). The high PDT or low PDT alone was discussed in the Fig. 9 and Fig. S9. Though the high PDT alone (2 or 3 min) obtained ultra-high PDT efficacy by GNR-PSS-PEI-mPEG-SH-AlPcS4, there was also significant viability loss by free AlPcS4. Decreasing the irradiation time (0.5 or 1 min) of high PDT alone could reduce the cell viability loss by free AlPcS4, but the therapeutic effect by GNR-PSS-PEI-mPEG-SH-AlPcS4 would be also reduced. Similarly, the low PDT alone could not obtain ultra-high PDT efficacy by GNR-PSS-PEI-mPEG-SH-AlPcS4, when the

therapeutic effect by free AlPcS4 had no significant cell viability loss. Additionally, the Fig. 9 and S10 indicated that (the effect of high PDT) $\times$ (the effect of low PDT) $<$ (the effect of high/low PDT mode), which could be attributed to the plasmonic photothermal release of AlPcS4.

Because the high/low PDT mode had two treatment modes (high PDT and low PDT), it was necessary to discuss the treatment interval between high PDT and low PDT. The previous experiments above were all continuous. Fig. 10 showed the results of different intervals from 0 s to 2 h. The conclusion was that the therapeutic effect of 0 s interval was higher than others ($*p < 0.05$). There were two possible reasons: (1) free AlPcS4 released from GNRs in the endosomes and lysosomes would diffuse to the outside of the cell as time increased, which reduced the PDT efficacy. (2) Localized high concentration of AlPcS4 in the cell would help improve the PDT efficacy due to free AlPcS4 released from GNRs in the endosomes and lysosomes diffusing to the whole cell with time.

In our current study, we investigated a highly efficient and safe photodynamic therapy procedure by developing a high/low PDT mode using mPEG-SH modified GNR-AlPcS4 complexes. The possible mechanism of high/low PDT mode was presented in Fig.11. Based on the high loading efficiency of GNR-AlPcS4 complexes, high power density light was firstly used to accelerate the release of AlPcS4 from the surface of GNRs through the plasmonic photo-thermal effect of GNRs. Simultaneously, the PDT was applied as single continuous wave laser was used. Next, low power density light was used to attain prolonged PDT efficacy through AlPcS4 release over a relatively long timeframe. Overall, this high/low PDT utilizing nanomaterials yielded high PDT efficacy (about 90% tumor cell killing). Applying the same methods in the control group (free AlPcS4), there was no significant cytotoxicity, because of lower cellular concentrations of AlPcS4 and insufficient irradiation doses given the species of photosensitizer. Only free AlPcS4 or the free AlPcS4 released from GNRs before entering the cells could diffuse into cells easily, including the normal cells, and thus this high/low PDT mode could effectively reduce the treatment risk and the cytotoxicity to normal cells, helping to realize a highly efficient and safe photodynamic therapy.

4. Conclusions

In summary, we reported a high/low PDT mode using mPEG-SH modified GNR-AlPcS4 complexes as an example. The high/low PDT mode (80 mW/cm², 0.5 min for high light dose and 25 mW/cm², 1.5 min for low light dose) achieved ultra-high PDT efficacy by 0.5 nM mPEG-SH modified GNR-AlPcS4 complexes (7 μ M AlPcS4 equiv.) (about 90% tumor cell killing) and no significant viability loss was induced by free AlPcS4 photosensitizer under the same irradiation conditions. Additionally, while ensuring the therapeutic effect, the high/low PDT mode reduced the use of high power density light and thus less treatment risk. Based on the simple polyelectrolyte coated GNRs, mPEG-SH was conjugated on the surface of GNRs to improve the stability, reduce the cytotoxicity and increase the encapsulation (near 100%) and loading efficiency (the molar ratio of bound AlPcS4 per GNR was about 14,000:1). By incorporating nanotechnology, this high/low PDT mode resulted in a highly efficient and safe photodynamic therapy procedure. Combined with

precise control of light distribution in tissues, the accurate, ultra-highly efficient, and safe photodynamic therapy procedure is expected to achieve based on this high/low PDT mode.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A schematic explaining the photodynamic therapy mode using a high/low power density photodynamic therapy (PDT) treatment with single laser irradiation is depicted. (a) Only low power density PDT. (b) Only high power density PDT. (c) High/low power density PDT.

Fig. 2.

A schematic representation for preparation of mPEG-SH modified GNR-AIPcS4 complexes is depicted.

Fig. 3.

The characterization of gold nanorods is shown. (a) UV-Vis-NIR absorption spectra of AIPcS4, GNR-CTAB, GNR-PSS, GNR-PSS-PEI, GNR-PSS-PEI-mPEG-SH and corresponding GNR-AIPcS4 complexes are presented. (b) A TEM image of GNR-PSS-PEI-mPEG-SH-AIPcS4 is shown. The average size (c) and the zeta potential (d) of GNR-CTAB, GNR-PSS, GNR-PSS-PEI, GNR-PSS-PEI-mPEG-SH and corresponding GNR-AIPcS4 complexes ($n = 3$) are given.

Fig. 4. AlPcS4 release from mPEG-SH modified GNR-AlPcS4 complexes with and without NIR laser irradiation in PBS solution is shown. The PBS outside the dialysis membrane was changed after each test.

Fig. 5.

The cytotoxicity of GNR nanoparticles in MCF-7 human breast cancer cells was measured by an MTT assay (incubation time = 24 h). Data were expressed as the mean $\pm$ standard ($n = 3$).

Fig. 6.

Confocal microscopy images of cancer cells incubated with free AIPcS4 (14 μM) and mPEG-SH modified GNR-AIPcS4 complexes (1 nM, 14 μM AIPcS4 equiv.) for 4 h are shown. Blue fluorescence showed Hoechst 33342 labeled nuclei, green fluorescence indicated FITC-phalloidine stained cell membrane, and red fluorescence was internalization of AIPcS4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 7.

Cell viability of MCF-7 cells incubated with GNR-PSS-PEI-mPEG-SH, free AIPcS4, AIPcS4+GNR-PSS-PEI-mPEG-SH and GNR-PSS-PEI-mPEG-SH-AIPcS4 (0.5 nM , $7 \text{ }\mu\text{M}$ AIPcS4 equiv.) is shown after high/low photodynamic therapy mode (High = 400 mW/cm^2 , 3 min; low = 200 mW/cm^2 , 5 min). Statistically significant differences were evaluated using the Student's *t* test (* $p < 0.01$, ** $p > 0.05$).

Fig. 8. Cell viability of MCF-7 cells incubated with free AIPcS4, AIPcS4+GNR-PSS-PEI-mPEG-SH and GNR-PSS-PEI-mPEG-SH-AIPcS4 (0.5 nM, 7 μ M AIPcS4 equiv.) after high/low photodynamic therapy mode is shown. (a) High = 80 mW/cm², 2 min; low = 25 mW/cm², 4 min. (b) High = 80 mW/cm², 1.5 min; low = 25 mW/cm², 2 min. Statistically significant differences were evaluated using the Student's *t* test (**p* < 0.05, ***p* > 0.05).

Fig. 9. Cell viability of MCF-7 cells incubated with free AlPcS4 and GNR-PSS-PEI-mPEG-SH-AlPcS4 (0.5 nM, 7 μ M AlPcS4 equiv.) after high/low photodynamic therapy mode is shown. High = 80 mW/cm², 0.5 min; low = 25 mW/cm², 1.5 min. High PDT alone 2 min and low PDT alone 2 min were discussed. Statistically significant differences were evaluated using the Student's *t* test (**p* < 0.01, ***p* > 0.05).

Fig. 10.

Cell viability of MCF-7 cells incubated with GNR-PSS-PEI-mPEG-SH-ALPcS4 (0.5 nM , $7 \text{ }\mu\text{M}$ ALPcS4 equiv.) at different intervals between high PDT and low PDT is shown. High = 80 mW/cm^2 , 20 s; low = 25 mW/cm^2 , 60 s. Statistically significant differences were evaluated using the Student's *t* test ($*p < 0.05$).

Fig. 11.

This schematic presents the possible mechanism of high/low PDT mode.