Photosensitizer-loaded Gold Nanorods for Near Infrared Photodynamic and Photothermal Cancer Therapy

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Keywords: gold nanorod, near infrared, photodynamic therapy, photothermal therapy, cancer

Abstract

Despite the advancement of photodynamic therapy and photothermal therapy, the ability to form compact nanocomplex for combined photodynamic and photothermal cancer therapy under a single near infrared irradiation remains limited. In this work, we prepared an integrated sub-100 nm nanosystem for simultaneous near infrared photodynamic and photothermal cancer therapy. The nanosystem was formed by adsorption of silicon 2,3-naphthalocyanine dihydroxide onto gold nanorod followed by covalent stabilization with alkylthiol linked polyethylene glycol. The effects of alkylthiol chain length on drug loading, release and cell killing efficacy were examined using 6-mercaptohexanoic acid, 11-mercaptopundecanoic acid and 16-mercaptohexadecanoic acid. We found that the loading efficiency of silicon 2,3-naphthalocyanine dihydroxide increased and the release rate decreased with the increase of the alkylthiol chain length. We demonstrated that the combined near infrared photodynamic and photothermal therapy using the silicon 2,3-naphthalocyanine dihydroxide-loaded gold nanorods exhibit superior efficacy in cancer cell destruction as compared to photodynamic therapy and photothermal therapy alone. The nanocomplex stabilized with 16-mercaptohexadecanoic acid linked polyethylene glycol provided highest cell killing efficiency as compared to those stabilized with the other two stabilizers under low drug dose. This new nanosystem has potential to completely eradicate tumors via noninvasive phototherapy, preventing tumor reoccurrence and metastasis.

Keywords: gold nanorod, near infrared, photodynamic therapy, photothermal therapy, cancer

Introduction

Photodynamic therapy (PDT) is a type of cancer treatment that uses noninvasive light. In PDT, photosensitizers (PSs) absorb photon energy and are then excited from ground states to high energy states which transfer energy to neighboring oxygen molecules, leading to production of high energy reactive oxygen species (ROS) (mainly singlet oxygen) to kill cancer cells.^[1] As the treatment is noninvasive, localized, portable, inexpensive, and simple to operate, it has received considerable attention for many years and used in the clinic for the treatment of certain cancers such as esophageal cancer and non-small cell lung cancer.^[2] However, conventional PDT is limited by the poor water-solubility and non-specificity of most PSs. To improve the delivery efficiency and tumor specificity, substantial efforts have been made on the development and utilization of nanocarriers during the past two decades.^[3]

A new generation of PS carriers is photothermal (PT) nanoplatform. The rationale is that cancer cells can not only be destroyed by PDT via the PS molecules but also photothermal therapy (PTT) through the photothermal NP carriers. Different from PDT, PTT utilizes the heat produced from photothermal agents, either dye molecules or more efficient light-absorbing nanoparticles (NPs) such as carbon nanomaterials^[4] and plasmonic gold nanoparticles^[5] under laser irradiation to physically destroy cancer cells. By combining PDT and PTT, cancer cells can be more effectively destroyed than the single treatment due to the additive or synergistic effects of the two treatments. For in vivo applications, the NPs require light absorption in the near infrared (NIR) window where light absorption by the tissue major absorbents water and hemoglobin is minimized.^[6] Graphene oxide,^[7] gold nanorods (Au NRs),^[8] and some other NIR-absorbing NPs and nano-assemblies^[9] have been used as PS carriers to enable dual mode PDT and PTT. Particularly, Au NRs have attracted a great deal of attention because of several advantages: (1) Small size (around 50 nm in length), (2) ease of preparation, (3) excellent stability (shelf life more than one year), and (4) long blood

circulation property, with the half-life more than 10 h when modified with polyethylene glycol (PEG).^[10]

Despite the significant advancement of PDT and PTT, the ability to formulate compact PDT-PTT nanocomplex with high efficiency of PS delivery remains limited. In addition, most PDT and PTT modalities are operated sequentially using two lasers with different wavelengths due to the absorption mismatch of the PSs and PT NPs. We hypothesize that a new nanocomplex that enables simultaneous PDT and PTT under a single NIR laser irradiation for highly efficient cancer treatment can be developed using PT and PS agents with similar absorption wavelengths in the NIR region. In this work, we have developed such nanocomplex by nonspecific adsorption of silicon 2,3-naphthalocyanine dihydroxide (SiNC) ($\lambda_{\text{max}} = 780 \text{ nm}$) onto Au NRs ($\lambda_{\text{max}} = 795 \text{ nm}$) followed by covalent stabilization with alkylthiol (AT)-linked PEG. PSs were trapped in the hydrophobic pocket of alkylthiol (AT)-linked PEG on the surface of Au NRs. Highly efficient PS release and cellular uptake were achieved through partition of the hydrophobic PSs between the nanocomplex and cell lipid membrane.^[11] We have demonstrated through *in vitro* studies that the nanocomplex allows for complete eradication of cancer cells with low intensity of NIR light, superior to PDT or PTT alone. The nanocomplexes have great potential to deliver high concentration of PS and PT agents into tumor, which may lead to complete ablation of tumors and thus prevent tumor reoccurrence and metastasis.

Materials and Methods

Materials: All chemicals were purchased from Sigma-Aldrich unless specified. NH_2 -PEG 5000 was purchased from Laysan Bio (Arab, AL).

Synthesis and Characterization of Au NRs: Au NRs were synthesized based on the well developed seed-mediated growth method by Murphy and EL-Sayed groups.^[12] In a typical procedure, small Au NPs (2 to 5 nm) were firstly synthesized as the seed solution by reducing

chloroauric acid (HAuCl_4 , 0.25 mM) in a CTAB aqueous solution (0.1 M) with ice-cold sodium borohydride (NaBH_4) (0.6 mM final concentration). A 100 mL of growth solution containing 0.1 M CTAB, 0.5 mM HAuCl_4 and 90 μM silver nitrate (AgNO_3) was prepared in a 23°C water bath, followed by injection of 0.7 mL of 80 mM ascorbic acid (AA). Then, 80 μL of the seed solution was injected and Au NRs were formed within 2 h. The as-prepared CTAB-capped Au NRs were purified by centrifugation and resuspended in water for further use. The absorption spectrum was measured using an Agilent UV-VIS-NIR absorption spectrometer. The morphology and size were characterized using a Joel JEM1200 EX II transmission electron microscopy (TEM). Hydrodynamic size and zeta potential were measured by dynamic light scattering (DLS) with a Brookhaven 90 plus particle size analyzer.

Synthesis and Characterization of AT-PEG: AT-PEG was synthesized by linking AT and PEG with amide bond in an organic phase.^[13] Briefly, MHA, MUA or MHDA was dissolved in 4 mL of anhydrous dichloromethane (DCM) to form a 1 mM solution, followed by addition of 1mg NH_2 -PEG 5000, 0.1 mg 4-(dimethylamino) pyridine (DMAP) and 0.8 mg DCC. The mixture was stirred overnight in the dark to allow the formation of amide bond between AT and NH_2 -PEG. After solvent evaporation, the residue was reconstituted in ultrapure water and dithiothreitol (DTT) was added to cleave disulfide bond that may form between the polymers. Small molecules were separated by centrifugal filtration (MWCO 3000, Millipore). MHA-PEG, MUA-PEG and MHDA-PEG were characterized by nuclear magnetic resonance (NMR) spectroscopy. For the NMR measurement, the sample was lyophilized in dark for overnight, followed by reconstitution with deuterated chloroform (CDCl_3). ^1H spectra were recorded using a Varian 500 MHz NMR spectrometer. ^1H spectra of MHA, MUA, MHUA, and NH_2 -PEG were collected for comparison.

^1H NMR (500 MHz, CDCl_3) for MHA: δ 1.33 (t, $J = 7.4$ Hz, 1H), 1.40-1.51 (m, 2 H), 1.59-1.69 (m, 4 H), 2.37 (t, $J = 7.4$ Hz, 2H), 2.53 (q, $J = 7.4$ Hz, 2 H), and 10.10 (br s, 1H); ^1H NMR (500 MHz, CDCl_3) for MUA: δ 1.29 (m, 12 H), 1.34 (t, $J = 7.6$ Hz, 1 H), 1.62 (m, 4 H),

2.36 (t, J = 7.6 Hz, 2 H), 2.53 (q, J = 7.6 Hz, 2 H), and 11.25 (br s, 1H); ^1H NMR (500 MHz, CDCl_3) for MHDA: δ 1.26 (m, -22 H), 1.34 (t, J = 7.4 Hz, 1 H), 1.62(m, 4 H), 2.34 (t, J = 7.4 Hz, 2H), 2.53 (q, J = 7.4 Hz, 2 H), and 11.12 (br s, 1 H); ^1H NMR (500 MHz, CDCl_3) for NH_2 -PEG: δ 1.80 (br s, 2 H), 2.92 (t, J = 5.2 Hz, 2 H), 3.38 (s, 3 H), 3.52 (m, 2 H), and 3.54-3.83 (m, multiple H); ^1H NMR (500 MHz, CDCl_3) for MHA-PEG: δ 1.34 (t, J = 7.2 Hz, 1 H), 1.41 (m, 2 H), 1.63 (m, 4 H), 2.18 (t, J = 7.2 Hz, 2 H), 2.54 (q, J = 7.2 Hz, 2 H), 2.66 (t, J = 7.2 Hz, 2 H), 3.38 (s, 3H), 3.44 (m, 1H), 3.47- 3.81 (m, multiple H), and 5.67 (s, 1 H); ^1H NMR (500 MHz, CDCl_3) for MUA-PEG: δ 1.28 (m, 12 H), 1.34 (t, J = 7.3 Hz , 1 H), 1.62 (m, 4 H), 2.17 (t, J = 7.3 Hz, 2 H), 2.41 (t, J = 7.3 Hz, 1H), 2.53 (q, J = 7.6 Hz, 2 H), 2.67 (t, J = 7.3 Hz, 1 H), 2.85 (t, J = 7.3 Hz, 2 H), 3.38 (s, 3H), 3.53 (m, 2 H), 3.58-4.20 (m, multiple H), and 5.35 (br s, 7 H); ^1H NMR (500 MHz, CDCl_3) for MHDA-PEG: δ 1.24-1.35 (m, 23 H), 1.60 (m, 4 H), 2.27 (t, J = 7.4 Hz, 2 H), 2.51 (q, J = 7.4 Hz, 2 H), 2.97 (t, J = 5.1 Hz, 1 H), 3.37 (s, 3 H), 3.49 (t, J = 5.1 Hz, 2 H), 3.55 (m, 2 H), 3.57-3.69 (m, multiple H), 3.78 (t, J = 5.1 Hz, 2 H), and 4.72 (s, 1 H).

SiNC Loading and Stabilization on Au NRs: SiNC was loaded onto Au NRs via electrostatic adsorption followed by covalent stabilization with AT-PEG.^[13] Firstly, SiNC was dissolved in dimethyl sulfoxide (DMSO) and mixed with Au NR aqueous solution (50, 000 SiNC: 1 Au NR) for 15 min. Then, AT-PEG was added (50,000 AT-PEG: 1 Au NR) and the solution was stirred for 40 min. The AT-PEG/SiNC/Au NRs were purified by three times of centrifugation (12,000 rpm, 20 min) and the residue was reconstituted with ultrapure water. The supernatant was collected and free SiNC was extracted with DCM. After solvent evaporation, SiNC was resuspended in DMSO and quantified by Raman spectroscopy using a portable Raman spectrometer (TSI, Shoreview, MN) to determine the amount of un-adsorbed SiNC. SiNC adsorption on Au NRs was also directly confirmed by measuring the SERS signals of the nanocomplexes. The nanocomplexes were further characterized by DLS to determine the hydrodynamic size and zeta potential.

The Stability of AT-PEG/SiNC/Au NRs in Phosphate Buffer Solution (PBS), Cell Culture Medium and Serum: AT-PEG/SiNC/Au NRs were dispersed in 1x PBS (pH 7.4, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ and 1.47 mM KH₂PO₄), RPMI 1640 cell culture medium (Mediatech, Inc., Manassas, VA) with 10% fetal bovine serum (FBS, Gemini Bio-Products, West Sacramento, CA) or 100% FBS to make 1 mL of 0.5 nM (concentration of Au NR) solution. The solution was shaken on an orbital shaker (600 rpm) to mimic blood circulation. At a specified time from 30 to 360 min, 200 μ L of the solution was withdrawn and centrifuged. The supernatant was collected and mixed with equal volume of DCM and stirred vigorously for 4 h. Then, the DCM layer was collected and the solvent was evaporated. SiNC was reconstituted and concentrated in DMSO and quantified by Raman spectroscopy.

SiNC Release from AT-PEG/SiNC/Au NRs under Cellular Environment: KB-3-1 head and neck cancer cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS and penicillin-streptomycin solution (100 U/mL penicillin, 100 U/mL streptomycin) at 37°C under 5% CO₂. The cells were seeded at a density of 8×10^3 cells per well into 96-well plates in triplicate. After 24 h, the medium was replaced with 100 μ L of fresh medium containing 0.1 nM AT-PEG/SiNC/Au NRs. After a specified time from 30 to 360 min, the cell culture medium was collected. SiNC in the medium were extracted with DCM, followed by solvent evaporation and resuspension with DMSO. SiNC was quantified by Raman spectroscopy. SiNC released was expressed as the percentage of SiNC inside cells compared to the total amount of SiNC from the added AT-PEG/SiNC/Au NRs.

Laser Irradiation and Cell Viability Assay: Head and neck KB-3-1 cells and breast cancer SK-BR-3 cells were seeded at a density of 8×10^3 cells per well into 96-well plates. After overnight, cells were washed with PBS and immersed in 100 μ L of medium containing the following formulations: (1) AT-PEG/Au NRs, (2) SiNC (DMSO as the solvent), and (3) AT-PEG/SiNC/Au NRs. These three groups represent PTT, PDT and PTT+PDT treatment,

respectively. Cells immersed in medium only were used as the control. For the groups (1) and (3) formulations, three concentrations, 0.1, 0.3, and 0.5 nM (in terms of Au NRs) were used to examine the effects of the dosage of these formulations on the treatment efficacy. For the group (2) formulation, a series of concentrations ranging from 2 to 12 μM were used to match up the concentration of adsorbed SiNC in each AT-PEG/SiNC/Au NR complex. After incubation for 2 h, cells were exposed to laser irradiation (Beam size: 8 mm. Power intensity: 0.55 W/cm^2) for 10 min with a 808 nm diode laser (Power Technologies, Little Rock, AR). Then, cells were washed with PBS and incubated in fresh medium for overnight before cell viability assay. The control cells were not subjected to laser irradiation. Cell viability was determined using the XTT toxicology assay. The cell viability was expressed as the percentage of live cells over that of the untreated control. The data are presented as the mean $\pm$ standard deviation of triplicate measurements.

Statistical Analysis: The statistical significance of the cell viabilities under different treatments and different concentrations were compared using the analysis of variance (ANOVA). A p-value < 0.05 was considered statistically significant in all cases. Multiple comparison procedures were used after obtaining a significant ANOVA result. A post hoc Scheffe method was applied to determine which means differed. The mean difference between treatments or concentrations was considered to be significant if the absolute value was greater than the minimum significant difference (MSD) that was derived from the Scheffe method.

Results and Discussion

Synthesis and Characterization of Au NRs, AT-PEG, and AT-PEG/SiNC/Au NRs. Au NRs was synthesized following the well known seed-mediated growth method, in which Au NRs were formed by anisotropic growth of Au seed of 2-5 nm in a growth solution containing Au precursor, CTAB capping material, AA reducing agent and AgNO_3 additive.^[12] The aspect ratio of the rods was controlled by adjusting the amount of AgNO_3 in the growth solution. To

maximize laser absorption, we made Au NRs with an aspect ratio of 4.1 using 90 μM AgNO_3 in the growth solution. The NRs were approximately 50 nm in length and 12 nm in width, with a surface plasmon resonance (LSPR) around 800 nm (**Figure 1A&B**). To examine the photothermal efficiency, we irradiate the particle solution with different concentrations and monitored the rise of the temperature of the solution. Under irradiation with a 808 nm diode laser with low intensity (0.55 W/cm^2), the temperature of 0.1, 0.3 and 0.5 nM Au NR aqueous solutions increased from 23°C to 43°C , 68°C and 82°C , respectively, indicating their great photothermal property (**Figure 1C**). Our previous studies showed that Au NRs in such dimension gave photothermal conversion efficiency around 75%.^[14] The as-prepared Au NRs were capped with positively charged cetyltrimethylammonium bromide (CTAB) in a bilayer structure along the long axis.^[15]

Figure 1. Absorption spectrum (A), TEM image (B) and photothermal profile (C) of Au NRs. The Au NRs had a LSPR at 795 nm and aspect ratio of 4.1. Under 808 nm light irradiation with intensity of 0.55 W/cm^2 , the temperature of the Au NR aqueous solution with 0.1 nM, 0.3 nM and 0.5 nM concentrations increased from 23°C to 43°C , 68°C and 82°C , respectively.

To stabilize nanoparticles loaded with hydrophobic PS molecules, amphiphilic molecules are needed to trap the drugs on the particle surface with its hydrophobic segment while making the nanocomplex water soluble with its hydrophilic external layer.^[11a] In our previous studies,^[13] we prepared an amphiphilic polymer by covalently linking a small molecule carboxyl-terminated AT, 11-mercaptoundecanoic acid (MUA), to NH_2 -PEG (MW 5000) and demonstrated its capability to stabilize paclitaxel-loaded Au NRs. In this work, MUA-PEG was used to stabilize SiNC-loaded Au NRs. Further, 6-mercaptohexanoic acid (MHA) and 16-

mercaptohexadecanoic acid (MHDA) were used to examine the effects of AT chain length on drug loading and release efficiencies. All MHA-PEG, MUA-PEG, and MHDA-PEG were prepared using the standard N,N'-dicyclohexylcarbodiimide (DCC)-activated coupling reaction in organic phase between NH₂-PEG 5000 and MHA, MUA or MHDA. NH₂-PEG bound to the carboxyl-terminated AT molecules via amide bond while leaving free thiol for binding to Au NRs. The success binding of NH₂-PEG and the three carboxyl-terminated AT molecules were confirmed by NMR spectroscopy. **Figure S1** shows the ¹H spectra of MHA, MUA, MHDA, NH₂-PEG, MHA-PEG, MUA-PEG, and MHDA-PEG with peak assignments. All the carboxyl-terminated AT molecules gave the characteristic S-H triplet around δ (chemical shift) =1.25-1.35 ppm.^[16] They also showed a broad COOH peak above 10 ppm. The NH₂-PEG shows a weak peak at δ =1.80 ppm from the primary amine.^[17] The multiple protons from -CH₂CH₂O- unit give strong peaks from 3.54 to 3.83 ppm. The ¹H NMR spectra of MHA-PEG, MUA-PEG and MHDA-PEG shows a combination of that from the carboxyl-terminated AT and NH₂-PEG, but with the disappearance of the -COOH and the appearance of a new peak from 4-6 ppm that was assigned to the secondary amine.^[18] The secondary amine results from the formation of the amide bond between MHA, MUA or MHDA and NH₂-PEG. These results confirm that these AT molecules were successfully linked to NH₂-PEG.

Desirable PS agent should absorb light in the tissue transparent NIR region. They should also have good singlet oxygen quantum yield ($\phi_{\Delta} \geq 0.2$) to ensure sufficient efficacy. In our studies, SiNC was used as the PS agents. SiNC has absorption around 780 nm in DMSO (**Figure 2**). It has good

singlet oxygen quantum yield ($\phi_{\Delta}=0.35$),^[19] comparable to that of the clinically used phthalocyanine (PC)-based molecules whose NIR absorption is marginal.^[11b]

Figure 2. Structure (insert) and absorption spectra of SiNC.

AT-PEG/SiNC/Au NRs were prepared using the method we developed previously.^[15] SiNC was nonspecifically adsorbed onto Au NRs through hydrophobic interactions with the CTAB bilayer, followed by stabilization with MHA-PEG, MUA-PEG or MHDA-PEG (**Scheme 1**). Excessive SiNC (50,000 SiNC/Au NR) was added to ensure saturated adsorption. MHA-PEG, MUA-PEG or MHDA-PEG covalently bond to Au NRs via Au-S bonds. Compared to other drug loading methods, this method is ease of preparation and does not require chemical modification of the drug molecules.

Scheme 1. Schematic illustration of the preparation of AT-PEG/SiNC/Au NRs.

SiNC adsorption was characterized by Raman measurement. **Figure 3A** shows the surface enhanced Raman scattering (SERS) spectra of MHA-PEG/SiNC/Au NRs, MUA-PEG/SiNC/Au NRs and MHDA-PEG/SiNC/Au NRs, in comparison to the Raman spectrum of pure SiNC. All three nanocomplexes showed strong SERS signals from SiNC, confirming the adsorption of SiNC onto Au NRs. To quantify the amount of SiNC in each complex, unadsorbed SiNC was extracted with anhydrous dichloromethane (DCM). After solvent

evaporation, SiNC was dissolved in DMSO and measured by Raman spectroscopy. Although Raman signals from free SiNC were much weaker than SERS signals on Au NRs, quantitative measurement of SiNC in solution was able to be obtained using elevated laser power and acquisition time. Using the quantitative relationship obtained from standard samples (**Figure S2**) and the Raman spectra collected from the un-adsorbed SiNC (**Figure 3B**), the amount of adsorbed SiNC on Au NRs was determined by difference for each AT-PEG. The results

Figure 3. Raman characterization of SiNC adsorption on Au NRs. (A) SERS spectra of MHA-PEG/SiNC/Au NRs, MUA-PEG/SiNC/Au NRs and MHDA-PEG/SiNC/Au NRs, in comparison with the Raman spectrum from free SiNC. (B) Raman spectrum of SiNC collected from un-adsorbed SiNC during preparation of the nanocomplex when MHA-PEG, MUA-PEG or MHDA-PEG was used as the stabilization agent. (C) Quantitative plot of the surface density of SiNC for MHA-PEG/SiNC/Au NRs, MUA-PEG/SiNC/Au NRs and MHDA-PEG/SiNC/Au NRs. Error bar is the standard deviation from triple experiments. (D) Schematic illustration of MHA-PEG/SiNC/Au NRs, MUA-PEG/SiNC/Au NRs and MHDA-PEG/SiNC/Au NRs. Laser wavelength: 785 nm. For the SERS measurement of the nanocomplexes, the laser power is 25 mW and the acquisition time is 1 s. For Raman measurement of free SiNC solution, the laser power is 250 mW and the acquisition time: 10 s. The concentration of free SiNC in (A) is 5 μM in DMSO.

showed that the number of SiNC per Au NR is 1.8×10^4 , 2.1×10^4 , and 2.4×10^4 for MHA-PEG, MUA-PEG and MHDA-PEG, respectively (**Figure 3C**). This demonstrates that the number of adsorbed SiNC increases as the number of carbon in AT segment increases. This is probably because the longer AT forms a larger hydrophobic pocket than the shorter AT on the surface of Au NR (**Figure 3D**). As a matter of fact, previous studies have shown that hydrophobic drugs can be trapped in the Au NP monolayer formed by MUA-linked tetra (ethylene glycol).^{11(a)} It is believed that the MUA segments form the hydrophobic pocket to trap the hydrophobic drugs, the ethylene glycol forms a hydrophilic external shell to stabilize the drug-loaded complex in aqueous solution. Our previous studies by high performance liquid chromatography (HPLC) have shown that such pocket can trap hydrophobic paclitaxel with $\sim 2 \times 10^4$ per Au NR.¹³ Here we used Raman spectroscopy as a simple characterization technique and found a similar loading capacity of SiNC to Au NRs covalently bound with MUA-PEG. Further, we found this drug loading capacity indeed depends on the alkanethiol chain length, with longer one leading to higher SiNC loading density. Due to the hydrophilic nature of PEG, we believe incorporation of SiNC in the PEG layer is trivial.

AT-PEG binding onto Au NRs was confirmed by DLS measurement. **Table 1** showed that the as-prepared Au NRs had the hydrodynamic diameter of 59 nm and zeta potential of 31 mV. The highly positive surface charge is due to the positively charged CTAB capping materials. The MHA-PEG/SiNC/Au NRs, MUA-PEG/SiNC/Au NRs and MHDA-PEG/SiNC/Au NRs had hydrodynamic diameter of 77, 79 and 82 nm, respectively. The surface charge became slightly negative. The nanocomplexes showed similar size and zeta potential to Au NRs

Table 1. Dynamic light scattering characterization of SiNC loading and stabilization.

	Hydrodynamic Size (nm)	Zeta Potential (mV)
CTAB/Au NRs	58.9 ± 1.8	31.2 ± 1.3
MHA-PEG/Au NRs	77.1 ± 2.1	-6.3 ± 1.1
MHA-PEG/SiNC/Au NRs	77.8 ± 2.3	-5.4 ± 1.2
MUA-PEG/Au NRs	79.1 ± 1.9	-10.2 ± 1.5
MUA-PEG/SiNC/Au NRs	79.4 ± 2.2	-8.6 ± 1.3
MHDA-PEG/Au NRs	82.2 ± 2.4	-9.3 ± 1.2
MHDA-PEG/SiNC/Au NRs	83.1 ± 2.0	-8.5 ± 1.4

modified with AT-PEG only. Thus, the changes on size and zeta potential after SiNC adsorption and AT-PEG binding resulted from the binding of AT-PEG onto Au NRs.

Stability and Drug Release Efficiency of AT-PEG/SiNC/Au NRs. The stability of each nanocomplex in physiological environments was examined using phosphate buffered saline (PBS), cell culture medium containing 10% serum and pure serum. To mimic blood circulation, the nanocomplexes in the above solutions were continuously shaken on an orbit shaker. At different time, an aliquot of the solution was centrifuged and the supernatant was treated with DCM extraction to collect SiNC released from the nanocomplexes. Again, SiNC was quantified by Raman spectroscopy. **Figure 4 (A-C)** shows the percentage of SiNC released at different time from MHA-PEG/SiNC/Au NRs, MUA-PEG/SiNC/Au NRs and MHDA-PEG/SiNC/Au NRs. The results showed that less than 2% of SiNC was released after 1 h for all the three complexes. After 6 h, around 8% of SiNC was released. These results demonstrate that all the AT-PEG/SiNC/Au NRs are stable in physiological environments, suitable for in vivo applications. The alkyl chain length of AT does not affect the stability of the SiNC-loaded Au NRs although it affects the loading capacity of SiNC on Au NRs.

It is known that hydrophobic molecules can enter cells via partition between nanocomplex and the apolar sites of the cell lipid membrane.¹¹ Thus, drug release is augmented by exposure of the nanocomplex to cell membrane. Although such a drug release mechanism could lead to drug uptake into normal cells, it is appropriate for the localized light-activated PTT and PDT. To quantitatively investigate this drug release and cellular uptake, we incubated SK-BR-3 cells with cell culture medium (with 10% serum) containing MHA-PEG/SiNC/Au NRs, MUA-PEG/SiNC/Au NRs or MHDA-PEG/SiNC/Au NRs. After a specified time, we determined the amount of SiNC remained in the medium by Raman spectroscopy. Accordingly, the amount of released SiNC represents the amount of SiNC uptake by the cells. **Figure 4D** shows the percentage of SiNC released at different time for the three nanocomplexes. The results showed that all three nanocomplexes exhibited a biphasic drug release profile with the rapid

Figure 4. Stability of MHA-PEG/SiNC/Au NRs (A), MUA-PEG/SiNC/Au NRs (B), and MHDA-PEG/SiNC/Au NRs (C) in PBS, cell culture medium and serum. (D) Comparison of the release properties of SiNC from MHA-PEG/SiNC/Au NRs, MUA-PEG/SiNC/Au NRs and MHDA-PEG/SiNC/Au NRs under cellular environment. Error bar is shown as the standard deviation from triple experiments.

release process occurring within the first two hours. The release efficiency is dependent on the structure of the AT-PEG stabilization agent. The release rate decreased when the AT chain length increased. The difference in the drug release efficiency is especially significant within the rapid release process. For examples, at 30 min, 50%, 39% and 17% of SiNC was released for MHA-PEG/SiNC/Au NRs, MUA-PEG/SiNC/Au NRs and MHDA-PEG/SiNC/Au NRs, respectively. At 2 h, 75%, 69% and 67% SiNC was released for MHA-PEG/SiNC/Au NRs, MUA-PEG/SiNC/Au NRs and MHDA-PEG/SiNC/Au NRs, respectively. At 6 h, all the three nanocomplexes showed similar efficiency of SiNC release (~ 80%). Thus, the amount of SiNC released is proportional to the amount of adsorbed SiNC. For a 0.1 nM nanocomplex,

the content of SiNC released from MHA-PEG/SiNC/Au NRs, MUA-PEG/SiNC/Au NRs, and MHDA-PEG/SiNC/Au NRs is estimated to be 1.44, 1.68, and 1.92 μM , respectively.

Photodynamic and Photothermal Therapy with AT-PEG/SiNC/Au NRs. To examine the capabilities of AT-PEG/SiNC/Au NRs for dual mode PDT and PTT of different types of cancers and the effect of the AT-PEG structure on the treatment efficacy, we comparably studied the cytotoxicity of PDT, PTT, and PDT + PTT of KB-3-1 and SK-BR-3 cells using each of MHA-PEG/SiNC/Au NRs, MUA-PEG/SiNC/Au NRs and MHDA-PEG/SiNC/Au NRs. PTT was achieved using these nanocomplexes without SiNC. We chose KB-3-1 and SK-BR-3 to represent light assessible head and neck cancer and breast cancer, respectively. Both types of cancers are assessible to light In these studies, cells were incubated with the specified drug formulations for 2 h and then exposed to laser for 10 min. The concentration of free SiNC for the PDT treatment in each dose was determined based on the SiNC loading efficiency and the release efficiency at 2h for each AT-PEG/SiNC/Au NR complex. After laser treatment (10 min, 0.55 W/cm²), cells were washed three times with PBS to remove drugs and then incubated in drug-free medium for overnight. XTT assay was used to determine cell viability. The studies were carried out under three doses (0.1, 0.3, and 0.5 nM Au NRs) or free SiNC with equivalent concentrations for both KB-3-1 and SK-BR-3 cells.

Figure 5 shows the cell viabilities under different treatments in two different cell lines. Statistical analysis with ANOVA followed by pair wise comparisons using Scheffe method showed significant differences ($P < 0.05$, Mean difference $> \text{MSD}$) between the combination treatment and individual treatments in both KB-3-1 and SK-BR-3 cells using any of the three AT-PEG stabilizer (**Table S1-21**). For examples, the cell viability under combined PDT and PTT using 0.1 nM MHA-PEG/SiNC/Au NRs was 17.3 % for KB-3-1 cells and 18.3% for Sk-BR-3 cells. However, the cell viability under PDT alone using equivalent SiNC (1.35 μM) was 58.4% for KB-3-1 cells and 59.9% for SK-BR-3 cells, and that under PTT alone

using equivalent

Figure 5. Comparison of the efficacies of PDT + PTT with AT-PEG/SiNC/Au NRs using KB-3-1 (left) and SK-BR-3 (right) cells at different doses to that of PDT alone with free SiNC and PTT alone with AK-PEG/Au NRs at equivalent concentrations. The results are mean values $\pm$ SD of triplicate experiments. (* $P < 0.05$ compared with PDT, PTT, and

PDT+PTT).

MHA-PEG/Au NRs (0.1 nM) was 40.7% for KB-3-1 cells and 33.9% for Sk-BR-3 cells. Thus, the combination treatment killed 41.1 % more KB-3-1 cells and 41.6 % more SK-BR-3 cells than PTT. It killed 23.4 % more KB-3-1 cells and 15.6 % more SK-BR-3 cells than PTT. These results show that combined PDT and PTT is significantly more effective than PDT or PTT alone. These significant differences were also observed in the treatments when MUA-PEG or MHDA-PEG was used as the stabilization agent. In terms of the combination treatment, the MHDA-PEG/SiNC/Au NRs showed slightly higher cell killing efficiencies than MHA-PEG/SiNC/Au NRs and MUA-PEG/SiNC/Au NRs in both cell lines. Based on the drug loading (Figure 3) and release efficiencies (Figure 4D), we calculated that 1.35 μ M SiNC was released from the 0.1 nM MHA-PEG/SiNC/Au NR, 1.45 μ M from 0.1 nM MUA-PEG/SiNC/Au NR, and 1.61 μ M from 0.1 nM MHDA-PEG/SiNC/Au NR when the nanocomplexes were exposed to cells for 2 h. Thus, the higher cell killing efficiency for MHDA-PEG/SiNC/Au NRs as compared to PEG/SiNC/Au NRs and MUA-PEG/SiNC/Au NRs was due to the higher amount of SiNC delivered into the cells by the MHDA-PEG/SiNC/Au NRs.

Increasing the concentration of the nanocomplex led to increased cell killing efficiencies for all groups. For example, the cell viability for the combination treatment decreased from 14.2% at 0.1 nM MHDA-PEG/SiNC/Au NRs to 4.9% at 0.3 nM for KB-3-1 cells. Further increase of the nanocomplex concentration to 0.5 nM led to complete cell destruction. Similar results were obtained in Sk-BR-3 cells. Increased drug concentration not only increased SiNC concentration but also the medium temperature. From Figure 1, we can see that the temperature of the medium was elevated to 43°C, 68°C and 82°C when 0.1 nM, 0.3 nM and 0.5 nM Au NRs were used, respectively. Thus, both PDT and PTT were amplified with the increase of the nanocomplex concentration. It is worthy to mention that complete

cell destruction was achieved under the combination treatment in both cell lines for all three nanocomplexes at the 0.5 nM concentration. These studies demonstrate that highly efficient cell killing can be achieved with 0.5 nM MHA-PEG/SiNC/Au NRs, MUA-PEG/SiNC/Au NRs or MHDA-PEG/SiNC/Au NRs in combination with low intensity laser (0.55 W/cm²) under combined PDT and PTT. Under this dosage, no significant difference was observed among MHA-PEG/SiNC/Au NRs, MUA-PEG/SiNC/Au NRs, and MHDA-PEG/SiNC/Au NRs.

Under elevated temperature due to the photothermal effect of Au NRs, cell membrane permeability may increase. This may increase drug diffusion and change the cellular uptake kinetics. Thus, a synergistic effect may exist between PDT and PTT, which will amplify the therapeutic outcome. To determine whether a synergistic effect exists in the combination treatment in our system, cell viability was compared to a purely additive result from PDT and PTT. The additive result was calculated using the relationship of $f_{\text{additive}} = f_{\text{PDT}} \times f_{\text{PTT}}$ [20], where f_{additive} is the fraction of surviving cells by additive interaction of PDT and PTT, f_{PDT} is the fraction of surviving cells resulting from PDT and f_{PTT} is the fraction of surviving cells resulting from PTT. When $f_{\text{combination}}$ is lower than f_{additive} , the effect of the two treatments is synergistic. When $f_{\text{combination}}$ is equal to f_{additive} , the two treatments have an additive effect. When $f_{\text{combination}}$ is larger than f_{additive} , they have an antagonistic effect. The calculated f_{additive} are listed in **Table 2**. The results showed that the $f_{\text{combination}}$ of PDT and PTT was significantly lower than the calculated f_{additive} in both KB-3-1 and SK-BR-3 cells in all three doses. Thus, a synergistic effect between PDT and PTT existed in both cell lines.

Table 2. Comparison of calculated f_{additive} with experimental $f_{\text{combination}}$ between PDT and PTT for KB-3-1 and SK-BR-3 cells.

Nanocomplex	Concentration (nM)	KB-3-1		SK-BR-3	
		f_{additive}	$f_{\text{combination}}$	f_{additive}	$f_{\text{combination}}$
MHA-PEG/SiNC/Au NRs	0.1	23.8	17.3	24.1	18.3
	0.3	10.5	9.9	9.8	8.4
	0.5	5.2	0	6.6	0

MUA-PEG/SiNC/Au NRs	0.1	17.5	17.0	19.5	15.4
	0.3	9.7	6.8	7.4	6.6
	0.5	3.9	0	4.5	0
MHDA-PEG/SiNC/Au NRs	0.1	16.7	14.2	17.9	14.4
	0.3	7.6	4.9	6.7	4.7
	0.5	3.7	0	2.9	0

Conclusion

Simultaneous PDT and PTT under tissue-penetrative NIR irradiation is highly desirable to maximize cancer phototherapy. Previously studies have demonstrated simultaneous PDT and PTT using indocyanine green (ICG)-adsorbed Au NR^[8a] or chitosan incorporated with ICG and Au NR.^[8b] ICG is hydrophilic and only has moderate singlet oxygen yield ($\phi_{\Delta}\sim 0.15$), which limits the therapeutic efficacy. The ICG-adsorbed Au NRs are impractical for in vivo tumor therapy as ICG is exposed on the surface of the nanocomplex. Incorporating Au NRs into the chitosan carrier limits the delivery efficiency of Au NRs as the loading efficiency of Au NRs is only 15%. In this work, we have made significant advances on the design of the nanocomplex. We utilize hydrophobic SiNC as the PS agent that has more than two times higher singlet oxygen yield than ICG. We use Au NRs as the carrier that can deliver more than 10^4 SiNC per particle by incorporating SiNC into the hydrophobic pocket of AT-PEG on the surface of Au NRs. Through the unique formulation, both Au NR and SiNC are protected by the stealthy PEG and thus the nanocomplex will have long circulation properties in the blood stream, ensuring sufficient tumor accumulation of both Au NR and SiNC.

Using two different cancer cell lines, we have tested the hypothesis that this AT-PEG/SiNC/Au NR complex allows simultaneous PDT and PTT under a single NIR laser irradiation and the therapeutic efficacy depends on the AT chain length. We demonstrated that the combined PDT and PTT were achieved under the laser irradiation at 808 nm, which has led to remarkably improved cell death as compared to PDT or PTT alone. By comparative studies using three AT-PEG molecules with different AT chain lengths (MHA-PEG, MUA-PEG and MHDA-PEG), we found that the cell killing efficiency of the nanocomplex

increased when the AT chain length increased due to the increased loading efficiency of SiNC. The cell killing efficiency was also dose dependent, with increasing concentration of the nanocomplex leading to higher percentage of cell death. Complete cell destruction under laser irradiation has been achieved with low density (0.55 W/cm^2) light and low concentration of AT-PEG/SiNC/Au NRs (0.5 nM).

Driven by the strong promises, our next work will be the investigation of the effects of AT chain length on the biodistribution and the delivery efficiency of SiNC into tumors. These studies are essential to determine the best AT-PEG/SiNC/Au NR formulation for tumor therapy. Then, we will test the capability of the nanocomplex for tumor therapy using head and neck cancer as a model. We envision that the MHDA-PEG/SiNC/Au NRs will be able to completely destroy tumors by dual PDT and PTT with a synergistic effect under a single NIR laser irradiation. Our method may prevent tumor regrowth and metastasis and will have important impact on the treatment of breast cancer, head and neck cancer, and other malignancies that are accessible by NIR light.

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Graphical Abstract