Self-assembled gold nanostar–NaYF₄:Yb/Er clusters for multimodal imaging, photothermal and photodynamic therapy†

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A grand challenge for medicine is to develop tools to selectively image and treat diseased cells. Rare earth doped upconverting nanoparticles (UCNPs) have been extensively studied for imaging applications because of their ability to absorb near infrared radiation (NIR) and emit visible light, but these particles cannot induce therapy alone. Recently, we developed methods to couple the UCNPs to visible and NIR-absorbing gold nanostructures through nucleic acid interactions. Here, we show that gold–UCNP clusters with optimized plasmon resonance and particle compositions provide both in vitro imaging contrast and combination cell killing through simultaneous photothermal (PTT) and photodynamic (PDT) therapy. PDT was induced by embedding singlet oxygen photosensitizers in silica shells on the UCNPs. Upon photoexcitation with 980 nm light, the NIR absorbing gold–UCNP clusters both increased the local temperature and generated singlet oxygen, increasing cell killing relative to either modality alone. The multifunctional polyethylene glycol (PEG) coated gold–NaYF₄:Yb/Er clusters exhibited high biocompatibility without irradiation but synergistic cell killing of MCF-7 cancer cells under light excitation. Finally, we also demonstrate that an optimal gold plasmon resonance is critical for minimizing absorbance overlap with the photosensitizers.

Introduction

Because upconverting materials tend to exhibit low background auto-fluorescence, high photostability, low toxicity, and tunable emission, upconverting nanoparticles (UCNPs) have garnered significant interest for nanomedicine applications. In addition to imaging, the emissive wavelengths of UCNPs can excite photosensitizers with matching absorption profiles that generate singlet oxygen capable of inducing cell toxicity via photodynamic therapy (PDT). In addition, NIR-responsive noble metal nanostructures have been utilized to promote hyperthermic responses in the body. Therefore, formulations of gold–UCNP nanoparticle clusters can impart both imaging and simultaneous PDT and photothermal (PTT) therapy in the local vicinity of diseased cells. However, because the non-resonant energy transfer processes between metal surfaces and UCNPs cause significant decreases in UCNP fluorescence, we recently developed strategies to control the number of Au conjugated to each UCNP to optimize interparticle energy transfer. In the prior study, gold nanostars (AuNSs) and nanoparticles (AuNPs) were coupled to silica coated UCNPs by DNA interactions. AuNSs were used because these structures exhibit three-dimensional anisotropy that can respond independently of light polarization, and the plasmon resonances are also easily tuned to reach the NIR region. Furthermore, by tuning the molar ratio of AuNP or AuNS to the UCNP, rapid 10–15 °C increases in temperature were observed under 980 nm excitation with minimal photoluminescence quenching. In doing so, we obtained highly emissive imaging agents still capable of localized heating by a single excitation wavelength, as compared to other systems such as graphene oxide–UCNPs which require additional 808 nm laser irradiation for PTT.

In this report, the photosensitizer zinc phthalocyanine (ZnPc) was loaded into the silica shells on the UCNPs in order to obtain highly emissive agents that are capable of both PTT and PDT in...
in vitro cell killing assays. ZnPc was utilized because the absorption spectra matched well with the emission wavelengths of the UCNPs to generate singlet oxygen for PDT. Next, polyethylene glycol (PEG) chains were conjugated to the nanoparticles to impart biocompatibility and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell assays were run using the UCNPs alone or the Au–UCNP clusters under NIR illumination. From these studies, we observed significantly higher cell death rates (22.85% higher compared with PTT alone) with combined PTT and PDT. It was also found however that the synergistic effect between the photosensitizers and the gold was minimized.

Materials and methods

Reagents and materials

PEG-SH (2000), l-ascorbic acid (98%), silver nitrate (99%), gold (m) chloride trihydrate (99.9+%), Triton X-100, yttrium(III) chloride (99.99%), ytterbium(III) chloride (99.99%), erbium(III) chloride (99.9%), oleic acid (90%), ammonium fluoride (99.99%), tetraethylorthosilicate (99%), 3-amino propyltriethoxysilane (99%), bis-p-sulfonatophenyl phenylphosphine dihydrate dipotassium salt (97%), cyclohexane (99.5%), Igepal CO-520, ammonium hydroxide solution (30–33%), propidium iodide (94%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 4′-6-diamidino-2-phenylindole (DAPI) were all bought from Sigma Aldrich. 5 nm and 10 nm gold colloid were bought from Ted Pella. Sodium borohydride, sodium hydroxide (98.8%), dimethyl sulfoxide (99.7%), tris(2-carboxyethyl) phosphine hydrochloride and succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) were bought from Thermo Scientific. 1-Octadecene (90%) was bought from Acros. Zinc phthalocyanine (95%) and fluorescein diacetate (97%) were bought form Alfa Aesar. DNA1 (5′-SH-C6-TTA TAA CTA TTC CTA AAA AAA AAA A-3′) and DNA2 (5′-SH-C6-TTT TTT TTT TTA GGA ATA GGT ATA A-3′) were bought from Integrated DNA Technologies, Inc. All the chemicals were used as received without further purification. Deionized water was used throughout the experiments.

Synthesis of 25 nm NaYF4:Yb,Er upconverting nanoparticles (UCNPs)

Monodisperse NaYF4:18%Yb,2%Er UCNPs were synthesized using a previously reported method. In a typical procedure, 0.1562 g YCl3, 0.0503 g YbCl3, and 0.0055 g ErCl3 were mixed with 6 mL oleic acid and 15 mL octadecene. The solution was slowly heated to 160 °C with vigorous stirring under argon for 50 min to form a homogeneous transparent solution, and then allowed to cool to room temperature. Then, a 10 mL methanol solution of NaOH (0.1 g) and NH4F (0.148 g) was added drop-by-drop and the solution was stirred for another 40 min. The solution was then slowly heated and degassed at 110 °C for 20 min, then refilled with argon and degassed three times totally. After that, the solution was heated to 300 °C within 10 min and reacted for 70 min under argon. After the solution was cooled back to room temperature, the products were precipitated from the solution with ethanol and then washed with ethanol and water (1:1) three times.

Synthesis of silica coated UCNPs

In a typical procedure, 1.5 mL oleic acid stabilized UCNPs (about 6.2 mg) in toluene was dried in 70 °C. After that, 2 mL cyclohexane was added, the solution was sonicated for 10 min. Then, 10 mL water which contains 0.1 g CTAB was added into the above solution and stirred vigorously for overnight to evaporate the cyclohexane, resulting in a clear CTAB–UCNPs water solution. For coating mesoporous silica onto UCNPs, 2.5 mL of the above CTAB–UCNPs solution was added to the mixture of 25 mL water, 3 mL ethanol and 40 μL 1 M NaOH solution. And then 500 μL 10% tetrathylorthosilicate (TEOS) ethanol solution was added into the above solution dropwise and the reaction was maintained for 14 hours. The as-obtained products were centrifuged and washed with ethanol three times.

Synthesis of DNA modified silica coated UCNPs

Briefly, 1.8 mg silica coated UCNPs were first reacted with 3-aminopropyltriethoxysilane (APTES) in ethanol (V_APTES: V_ethanol = 5:95) for 5 h. Then the amine modified silica coated UCNPs (in 100 μL H2O) was reacted with 1.8 mg succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) in 500 μL DMSO solution for 5 h. The mixture was centrifuged and washed three times, then it was resuspended in 500 μL DMSO solution. 187 μL 200 μM of DNA2 (DNA2 : UCNP = 200 : 1) was mixed with 30 μL tris(2-carboxyethyl) phosphine (TCEP) (50 mM in 200 mM NaHCO3), then added to the SMCC modified UCNPs. The reaction was allowed to proceed overnight. Finally, the above products were purified and dispersed in water for further use. The procedure used for preparation of DNA modified 520 nm AuNPs, 700 nm AuNSs and 980 nm AuSSs is the same with our previous methods.

Preparation of ZnPc loaded Au–UCNP PEGylated clusters

Firstly, 45 mL DMSO solution which contained 2 mg ZnPc was prepared. Then, 15 mL ZnPc solution was mixed with each kind of Au–UCNP clusters and was sonicated for 5 min then shake for 24 hours. After which, the products were spun by centrifugation at 10 000 rpm for 12 min. The supernatant was carefully discarded and the pellet was washed by ethanol and PBS for five times. Then 4 mg HS-PEG2000 was added into each tube and the solution was then reacted for 12 hours. The PEG modified clusters were then centrifuged and washed with water for three times. The final samples were kept in 4 °C for further use.

Cell culture

The MCF-7 cells were grown to confluence at 37 °C and with 5% CO2 in Eagle’s Minimum Essential Medium (EMEM) medium containing 10% fetal bovine serum and 1% penicillin/streptomycin.

Cytotoxicity assay

The viability of MCF-7 cells were determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MCF-7 cells were seeded into a 96-well cell culture plate
at $10^4$ per well and then incubated for 24 h at 37 °C under 5% CO$_2$. Then, Au–UCP clusters dispersed in PBS were added to each well to give final particle concentrations of 0, 10, 40, 100 and 250 μg mL$^{-1}$. The cells were then incubated for 4 h at 37 °C under 5% CO$_2$. After incubation, particle containing media was removed, wells were washed using PBS to remove the non-uptaken particles and 90 μL of fresh medium was added. Then 20 μL of filter-sterilized MTT reagent [5 mg mL$^{-1}$ in PBS] was added to each well, and the plates were incubated at 37 °C for 4 h. After incubation, medium was removed and the precipitated formazan crystals were dissolved by adding acidic isopropyl alcohol (40 mM HCl). Absorption values of the dissolved formazan crystals in each well were measured at 570 and 750 nm using a microplate reader. All the samples were prepared in triplicate.

### Photothermal test

MCF-7 cells were incubated with 250 μg mL$^{-1}$ of Au–UCNP clusters for 4 hours, and excess Au–UCNP clusters were removed by PBS washing. Then, 980 nm laser (laser spot size: 1 mm in diameter; laser power: 500 mW; laser confluence: 15.9 W cm$^{-2}$) was used to irradiate the cells for 15 min and then replaced with fresh culture medium and cultivated for 12 h at 37 °C in order to determine the effects of the 980 nm laser irradiation and laser induced $^{1}$O$_2$ on cell viability. Cell viability was determined by MTT assay (as described above). All the samples were prepared in triplicate.

### Confocal fluorescence imaging

250 μg mL$^{-1}$ of Au–UCNP clusters were incubated with MCF-7 cells ($10^4$ per well) for 4 hours at 37 °C under 5% CO$_2$. Then the wells were washed by fresh PBS three times and fixed with 4% paraformaldehyde for 15 min. After that the nuclei were stained with 2 μg mL$^{-1}$ 4′,6-diamidino-2-phenylindole (DAPI) for 15 min and then the wells were washed with PBS three times. Luminescence signals were collected in the wavelength regions of 500–550 nm and 570–620 nm.

### Fluorescein diacetate (green)/propidium iodide (red) (FDA/PI) double staining

The hyperthermic and $^{1}$O$_2$ induced potential effect of Au–UCNP clusters on MCF-7 cells was roughly checked by monitoring the changes of lysosomal membranes. The Au–UCNP clusters (250 μg mL$^{-1}$) incubated MCF-7 cells were irradiated by the same laser for 15 min and then replaced with fresh culture medium and cultivated for 12 h at 37 °C. After that, the cell culture medium was removed and the cells were washed by PBS. Then, 100 μL staining solution was added to each wells and incubated for 10 min in the dark. At last, the staining solution was removed and the cells were washed by PBS three times. The samples were then checked by fluorescent microscopy. FDA/PI staining solution: 50 μL 2 mg mL$^{-1}$ PI PBS solution and 8 μL 5 mg mL$^{-1}$ FDA acetone solution were added into 5 mL PBS solution, the mixture should be freshly prepared and kept in dark.

### Results and discussion

NaYF$_4$:Yb/Er upconversion nanoparticles (UCNPs) were first synthesized using a thermal decomposition method to produce monodisperse nanocrystals ~25 nm in diameter (Fig. 1). Since the as-prepared NaYF$_4$:Yb/Er UCNPs are capped with oleic acid, the particles were first transferred into water using cetyltrimethylammonium bromide (CTAB) [Fig. S1 and S2, ESI†]. Next, to increase stability in biological media and permit photosensitizer loading onto the UCNPs, mesoporous silica shells ~10 nm thickness were condensed around the UCNPs, followed by removal of CTAB through treatment with ammonium nitrite [NH$_4$NO$_2$] at 60 °C for 2 h followed by washing with ethanol. In order to conjugate the gold nanoparticles (AuNPs) and gold nanostars (AuNSs) to the UCNPs, the silica-coated UCNPs were further reacted with aminopropyltriethoxysilane (APTES) in ethanol for 5 hours followed by N-hydroxysuccinimide (NHS)-maleimide (SMCC) in DMSO. The SMCC-modified UCNPs were then reacted overnight with thiol-terminated DNA oligonucleotides to produce DNA-modified UCNPs. After removal of excess DNA by centrifugation, UV-Vis absorption measurements of the supernatant showed that ~71 DNA strands were attached per UCNP. Concurrently, three kinds of DNA-conjugated Au nanostructures with plasmon resonances at 520 nm, 700 nm, and 980 nm were prepared using our previously published procedures (Fig. 2). DNA-stabilized Au nanostructures were then hybridized to the DNA-modified UCNPs using 1:10 Au:UCNP molar ratios (Scheme 1). These loadings had previously been shown to minimize photoluminescence quenching from the UCNPs. As shown in Fig. 2, TEM analysis showed that the AuNPs and AuNSs were well conjugated to the UCNPs, with very few unbound AuNPs and AuNSs. With the AuNSs, most of the interactions with the UCNPs appeared to occur at the tips of the AuNS branches, which can be attributed to the high anisotropy of the AuNSs. Next, in order to improve biocompatibility with cells, the Au–UCNP clusters were reacted with 5 mg thiolated-PEG$_{2000}$ overnight in water solution, after which the excess PEG was removed by centrifugation. While the presence of DNA on the Au and UCNPs could potentially prevent or decrease the amounts of PEG conjugated to the nanoparticles, as will be discussed in more detail below, in vitro cell assays showed that coating the nanoparticles with PEG did improve cell viability.

In order to evaluate the Au–UCNP clusters as imaging and therapy agents, we first measured the relative cell cytotoxicity of the UCNPs and Au–UCNP clusters against breast cancer MCF-7 cells. Fig. 1 TEM images of as-synthesized UCNPs and silica coated UCNPs.
cells in the absence of any photoirradiation. For this, a typical MTT cell viability assay was performed by first incubating the UCNP and Au–UCNP clusters with the cells at concentrations up to 250 μg mL⁻¹ for 4 h. Next, filtered MTT solutions in PBS were added to the wells and incubated for another 4 h, after which the media was carefully removed and mixed with 100 μL acidic isopropyl alcohol (IPA, 40 mM HCl). The mixtures were gently agitated at room temperature overnight to completely dissolve the dye crystals, followed by absorbance measurement on a plate reader. As shown in Fig. 3, by coating the UCNP and Au–UCNP nanoparticles with PEG, the overall cell viability greatly improved as compared to particles with no PEG attached. Coating nanoparticles with PEG both reduces the nonspecific binding of blood proteins and macrophages to nanoparticles while potentially increasing the particle circulation life-time for \textit{in vivo} applications.\textsuperscript{47,48} At the highest nanoparticle concentrations tested (250 μg mL⁻¹), both the PEGylated UCNPs and Au–UCNPs retained cell viabilities greater than 88% after 4 h incubation. In comparison, particles without PEG produced lower cell viabilities at only 56% after 4 h (Fig. S3, ESI†).

Next, we investigated the PEGylated Au–UCNP clusters as imaging agents. As mentioned earlier, clusters of 1:10 Au:UCNP demonstrated strong visible emission under 980 nm excitation.\textsuperscript{23} For the imaging studies in this work, the PEGylated UCNP and Au–UCNP clusters were cultured with MCF-7 cells for 4 h at 37 °C under 5% CO₂ using nanoparticle concentrations of 250 μg mL⁻¹.\textsuperscript{49} Excess nanoparticles were removed and the cells were prepared for imaging studies by fixing the cells with 4% paraformaldehyde for 15 min.\textsuperscript{49} The UCNP and Au–UCNP treated cells were then imaged by confocal microscopy using 405 nm and 980 nm excitation. As shown in Fig. 4 and Fig. S6 (ESI†), the cell nuclei were easily identified by 4′,6-diamidino-2-phenylindole (DAPI) staining. The quantified photoluminescence intensity of the images in Fig. 4 is shown in Table S1 and Fig. S7 (ESI†). Under 980 nm light, both the UCNPs alone and the Au–UCNP clusters showed strong visible emission in the green, with weaker fluorescence in the red; this emission profile was consistent with photoluminescence studies run previously.\textsuperscript{23,35,50} Since no autofluorescence was observed from the cells upon NIR excitation,\textsuperscript{51} the Au–UCNP clusters show clear promise as agents for both imaging and PDT.

Next we coupled UCNPs to NIR-absorbing Au to study the effect of simultaneous PDT and PTT, since it was previously demonstrated that combined therapy treatments could not only increase efficacy but could also lower toxic side effects in comparison to two light source cancer therapies.\textsuperscript{18,22,32,33} The spectral overlap between the UCNP emission with the absorbance profile of zinc phthalocyanine (ZnPc) allows a series of energy transfers to convert NIR absorption into singlet oxygen generation (\(^1\text{O}_2\)), which can induce cell cytotoxicity by \(^1\text{O}_2\)-induced apoptosis (Fig. 5 and Scheme 1).\textsuperscript{9,12,13} First, ZnPc was incubated with the mesoporous silica coated UCNPs in DMSO for 24 h, followed by repeated centrifuge washing to produce a blue-purple UCNP solution, demonstrating successful ZnPc incorporation within the silica shells. The dye 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA) was used to measure the amount of singlet oxygen production upon 380 nm excitation (Fig. S4 and S5, ESI†). Upon singlet oxygen

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Fig. 3  \textit{In vitro} cell viability results of MCF-7 cells incubated with Au–UCNP clusters at different concentrations (0, 10, 40, 100 and 250 μg mL⁻¹) for 4 h.

![Scheme 1](image-url)
generation, the ABDA fluorescence becomes quenched, and this decay can be measured and plotted as a function of irradiation time to NIR laser (Fig. 5).12

The applicability of the Au–UCNP clusters for both PDT and PTT was next assessed in in vitro cell assays. For this, PEGylated UCNPs were incubated with MCF-7 cell cultures for 4 h at 37 °C followed by replacement of cell media with fresh PBS. The UCNP treated cells were then photoirradiated for 15 min using a 980 nm laser (15.9 W cm−2), followed by MTT assays to measure cell viability. As shown in Fig. 6, in the absence of photoirradiation more than 90% of the cells remained viable in the presence of the UCNPs, which was consistent with the previous toxicity studies (Fig. 3). Conversely, more than 86% of the cells remained viable under photoirradiation without UCNPs, indicating that the NIR laser light itself did little to harm the cells. However, photoirradiation of MCF-7 cells with ZnPc-loaded UCNPs caused cell viability to decrease significantly to 78.05% and 44.08% for 40 μg mL−1 and 250 μg mL−1 concentrations, respectively (Fig. 6). These results therefore show the utilization of the UCNPs as agents for PDT with NIR excitation.

The effect of PTT alone was studied via the incubation of MCF-7 cells with the AuNP–UCNP and AuNS–UCNP clusters (without ZnPc) at concentrations of 250 μg mL−1, followed by irradiation for 15 min with 980 nm laser light. As shown in Fig. 6, while only about 10% cell death was observed from the cells treated with the AuNP–UCNP clusters (Au plasmon at 520 nm), for the 700 AuNS–UCNP (Au plasmon at 700 nm) and 980 AuNS–UCNP (Au plasmon at 980 nm) treated cells, cell viability sharply dropped to 42.44% and 51.02%, respectively. This matches the results from our previous work, which demonstrated rapid temperature gains from the AuNS–UCNP materials but almost no increase from the AuNP–UCNP system.23

Finally, the effect of the combined synergistic PDT and PTT effects on cell viability was illustrated by treating the MCF-7 cells with ZnPc loaded AuNP–UCNP and AuNS–UCNP clusters, followed by the same irradiation conditions. MTT assays showed that combined PDT and PTT led to significant gains in cell killing, as the cell viabilities dropped substantially to 70.17%, 19.59%, and 30.68% for the AuNP–UCNP, 700 AuNS–UCNP, and 980 AuNS–UCNP, respectively (Fig. 6). Thus combination therapy led to approximately 20% more cell death than PTT alone. Because of the partial spectral overlap between the ZnPc and the AuNPs (520 nm plasmon), less of the emitted light from the UCNPs was available for the ZnPc photosensitizer,
which accounts for the ~70% viability for these cells as compared to ZnPc-loaded UCNPs alone (44.08%). However, with the NIR responsive AuNS–UCNP clusters, significant increases in cell death were induced, presumably both to the limited overlap in optical signatures between the AuNS and ZnPc as well as the combined utilization of PTT and PDT. In addition, larger cell death was induced with the 700 AuNS–UCNP as compared to the 980 AuNS–UCNP which we can attribute to the larger increases in temperature caused by the 700 AuNSs than the 980 AuNSs upon NIR absorption.23 In addition to MTT assays, live-dead cell assays were also run by using fluorescein diacetate and propidium iodide.18 As shown in Fig. 7, the cell staining assays matched the results obtained from the MTT studies. Lastly, to assess the ability of using the Au–UCNP clusters as potential imaging agents in vivo, a piece of 3% agarose was placed on top of the wells of the MCF-7 cultures incubated with the 700 AuNS–UCNPs followed by confocal microscopy. As shown in Fig. 8, the UCNPs showed strong emission through the agarose gel demonstrating the possibility of utilizing the AuNS–UCNP clusters as theranostics, tissue engineering and cellular imaging.

Conclusions

In summary, a multifunctional nanotheranostic has been designed for PL imaging, PTT and PDT. We first show that attaching PEG chains to the UCNP and Au–UCNP clusters imparted high biocompatibility. Furthermore, the AuNP–UCNP and AuNS–UCNP still demonstrated strong emission in vitro cell assays through careful loading of the AuNP and UCNP components. We showed that NIR laser light was absorbed by the Au–UCNP clusters and transferred into local photothermal energy to induce cell cytotoxicity. By loading the photosensitizer ZnPc into the silica coatings on the UCNPs, singlet oxygen could also be generated to cause cell death. Finally, we demonstrate in this work that the ZnPc loaded AuNS–UCNP clusters could synergistically induce both PTT and PDT to effectively cause a 20% increase in cell death as compared to using either modality alone. These results therefore show the potential of using multifunctional nanotheranostics as highly efficient agents for tumor eradication.

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