Structure design and applications of upconversion nanoparticles

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Recently appeared inorganic photoswitchable upconversion nanoparticles (PUCNPs) become a competitive candidate to surmount the widespread issue of the organic counterparts - photobleaching. However, current PUCNPs follow solely Yb\(^{3+}/\)Nd\(^{3+}\) co-sensitizing mode, which results in complex multi-layer doping patterns and imperfectness of switching in UV-blue region. We have here adopted a new strategy to construct Nd\(^{3+}\) free PUCNPs - NaErF\(_4@\)NaYF\(_4@\)NaYbF\(_4\): 0.5\%Tm@NaYF\(_4\), in which a prominent UV-blue emission from Tm\(^{3+}\) is turned on upon 980 nm excitation which can be completely turned off by 800 nm light. The quasi-monochromatic red upconversion emission upon 800 nm excitation - a distinct feature of undoping NaErF\(_4\) upconversion system - endows the PUCNPs with promising image-guided photon induced “off-on” therapy in biomedicine. As a proof-of-concept we have demonstrated the imaging-guided photodynamic therapy (PDT) of cancer, where 800 nm excitation turns off the UV-blue emission and leaves the emission at 660 nm for imaging. Once the tumor site is targeted, excitation switching to 980 nm results in UV blue emission and the red emission. The former is used to induce PDT whereas the latter is to monitor the therapeutic process. Our study implies that this upconversion photoswitching material is suitable for real-time imaging and image-guided therapy under temporal and spatial control.

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4.1 Introduction

Photoswitchable emission applications have been attracting global research interest which stems from the emerging applications in ultrahigh-density optical data storage, optoelectronic devices, chemical sensing and especially biomedicine.\(^1\)\(^-\)\(^4\) Till now, most of the photoswitchable materials are based on organic fluorescent molecules and their derivatives, whose photostability is always a concern due to the inherent photobleaching.\(^5\)\(^-\)\(^7\) As an alternative, multi-layer doping nanostructures based on lanthanide-doped upconversion nanoparticles (UCNPs) possessing narrowband excitation and multi-band emission of lanthanide ions are proposed to be highly photostable and photoswitchable.\(^8\)\(^-\)\(^12\) Typically, UCNPs have the capability to convert near-infrared excitation light to ultraviolet (UV)-visible emission upon sequential multiphoton absorption or energy transfer, which is very attractive in biomedicine since it may lead to deep tissue penetration, minimal autofluorescence background, and low photodamage of tissue.\(^13\)\(^-\)\(^15\) Benefit from the developed ~800 nm excitable Nd\(^{3+}\)/Yb\(^{3+}\) co-sensitized upconversion (UC) system\(^16\)\(^-\)\(^18\) and ~980 nm excitable Yb\(^{3+}\)-sensitized UC system, in these years, the lanthanide-doped photoswitches have been successfully applied in inducing chemical reactions and others, such as switching of organic compounds,\(^10\)\(^,\)\(^19\) reversible regulation of cell-molecules interaction,\(^20\) reversible handedness inversion\(^12\) and orthogonal excitations-emissions co-regulated therapy.\(^11\)\(^,\)\(^21\)

However, limited by the doping elements of suitable spectroscopic properties, all the reported UC photoswitches till now are restricted to Nd\(^{3+}\) and Yb\(^{3+}\) co-sensitizing materials,\(^22\) which are complex in structure since it requests different doping patterns in at least five layers (see Figure 4.4b), and accompanied with a leakage in switch function-certain emission region cannot be completely closed (see Figure 4.5).\(^10\)

These restrictions can be lifted with recent advances in doping approaches of UCNPs. In 2017, Almutairi \textit{et al.}\(^23\) Liu \textit{et al.}\(^24\) and our team\(^25\) have independently reported that an effective UC luminescence can be realized in undoping NaErF\(_4\)@NaYF\(_4\) (or NaLuF\(_4\), NaGdF\(_4\) shell \textit{etc.}) nanostructures. Compared with conventional sensitizer/activator co-doping systems, this type of material possesses very interesting luminescence properties, \textit{e.g.} prominent quasi-monochromatic red UC emission of Er\(^{3+}\) and much weaker emission in UV-Visible region,\(^23\)\(^-\)\(^25\) owing to the ladder-like energy levels of Er\(^{3+}\), the robust Er\(^{3+}\)-Er\(^{3+}\) cross relaxation and the suppression of concentration quenching effect by shell protection, which shed light on developing high quality and less complex photoswitchable UC nanostructures.

In this work, we have successfully realized a complete photoswitching in UV-blue region with a four layers structure: NaErF\(_4\)@NaYF\(_4\)@NaYbF\(_4\): 0.5%Tm@NaYF\(_4\), in which the monochromatic red emission occurs upon ~800 nm excitation, whereas strong UV and blue emissions, besides the red emission, appear upon ~980 nm excitation (Figure 4.1). Compared with the reported photoswitching UCNPs, our design is (i) Nd\(^{3+}\)-free, which has only one doping layer avoiding complex doping pattern in multiple layers and simplifies the synthesis, and (ii) a complete photoswitching of Er\(^{3+}\) in UV-blue region (\textit{e.g.} ~380 nm, ~410 nm) due to the robust Er\(^{3+}\)-Er\(^{3+}\) cross relaxation in the un-doped NaErF\(_4\) core, which ensures the UV-blue emission coming solely from the UC emission of Tm\(^{3+}\). Given by this, the photoswitchable upconversion nanoparticles (PUCNPs) offer an alternative strategy for imaging-guided “off-on” phototherapy. More importantly, the nanoplatorms based on the PUCNPs could in principle circumvent the
harmful effect of photodamage caused by the “always ON” UV-blue light during diagnosis, which highlights the bio-applications of lanthanide-based UC nanomaterials.

**Figure 4.1** (a) The core-multishell structure of PUCNP. (b) Illustration of the composition of the PUCNP and its energy level diagrams under 980 nm and 800 nm NIR excitations, respectively. The designed structure results in wavelength-dependent emissions in the luminescent NaErF$_4$ core and NaYbF$_4$: Tm shell under dual NIR excitations.

### 4.2 Results and discussion

#### 4.2.1 Basic characterization of nanostructures

The general core-multishell nanostructure of PUCNPs is a prerequisite to ensure the realization of such photoswitching function. The as-designed NaErF$_4$@NaYF$_4$@NaYbF$_4$:0.5%Tm@NaYF$_4$ (C-S1-S2-S3, “C” means core, “S” means shell) nanostructure was synthesized by successive deposition of epitaxial multi shells. Figure 4.2a, b present the transmission electron microscopy (TEM) images of the PUCNPs, ensuring the uniform size and morphology of the nanoparticles with an average diameter of ~ 40 nm. Furthermore, the high-angle annular dark field scanning transmission electron microscopy (HAADF-STEM) images shown in Figure 4.2c confirm the core-multishell structure of PUCNPs. In HAADF-STEM imaging the brightness of the imaging point is proportional to the square of the atomic number of the on-site element. Heavier lanthanide elements (Er and Yb, brighter parts) and the lighter ones (Y, darker parts) of the nanoparticle can thus be well distinguished. The diameter of the core and the thickness of each shell are confirmed in Figure 4.2d to be around 15 nm, 4.5 nm, 3.5 nm, 4.5 nm, respectively. Furthermore, the EDS (energy dispersive spectrometer) mapping (Figure 4.2e) and electron energy loss spectroscopy (EELS, Figure 4.2f) results reaffirm the lanthanide ions distributions in such PUCNPs, in agreement with the result of Figure 4.2c. Both were measured on Tecnai G2 F20 S-TWIN D573 transmission electron microscope, where EDS measures the characteristic emission of elements under the X ray radiation, whereas EELS calculates the energy loss of incident electron beam which is induced by the inelastic scattering of atoms. Finally, the hexagonal phase of the obtained PUCNPs is confirmed by X-ray diffraction (XRD) pattern (Figure 4.3).
Figure 4.2 (a) TEM image of PUCNPs. (b) High-resolution TEM image of single PUCNP. (c) HAADF-STEM image of PUCNPs. (d) The magnified HAADF-STEM image of PUCNP for shell thickness measurement. (e) The HAADF-STEM-EDS mapping image of randomly selected PUCNP. (f) The electron energy loss spectroscopy (EE LS) analysis results of six PUCNPs, the scale bars in (f) are 50 nm.

Figure 4.3 XRD patterns of NaErF₄ (core), NaErF₄@NaYF₄ (C@S1), NaErF₄@NaYF₄@NaYbF₄: Tm (C@S1@S2), and NaErF₄@NaYF₄@NaYbF₄: Tm@NaYF₄ (C@S1@S2@S3). All the diffraction peaks can be assigned to the standard card (JCPDS: 16-0334), indicating the successful formation of β-NaREF₄ nanoparticles.
4.2.2 Spectroscopic properties of PUCNPs nanostructure

Figure 4.4 (a) Upconversion emission spectra of NaErF₄@NaYF₄@NaYbF₄:0.5%Tm@NaYF₄ PUCNPs under 800 nm or 980 nm excitations at 5 W/cm². The insert photograph shows the PUCNPs in cyclohexane solution under irradiation of two laser beams of 0.8 W/cm², (b) structure and photoswitch principles of NaErF₄-based PUCNPs in this work and the conventional Nd-based ones, (c) UC luminescence spectra of different shell thicknesses of NaErF₄@NaYF₄, (d) UC luminescence spectra of the NaYF₄@NaYF₄: x% Yb, 0.5%Tm@NaYF₄(x:20-99.5) core-shell-shell nanoparticles as a function of Yb³⁺ content in the middle layer. Excitation is CW 980 nm laser of 5 W/cm². Inset: the Yb³⁺ concentration dependent UC emission intensity (integrated from 250 nm to 500 nm) of each type of core-shell-shell nanoparticles.

The corresponding photoswitching properties of the PUCNPs are displayed in Figure 4.4a. Upon excitation at 800 nm, only the monochromatic red emission (~ 660 nm) appears in the UV-visible region originated from the NaErF₄@NaYF₄ core-S1 part. Interestingly, upon 980 nm excitation, the UC red emission from the core-S1 part remained and the UV-blue emissions resulting from the S2-S3 part (NaYbF₄:0.5%Tm@NaYF₄) appear—a typical photoswitching process. As shown in Figure 4.4b, compared with the previously reported photoswitch structures, the PUCNPs not only simplify the structure by avoiding the Nd³⁺-doped layer and complex doping patterns in different layers, but also offer a better spectral regulation in the UV-blue region. All these advantages are attributed to the introduction of the Er³⁺-based self-sensitized UC system (NaErF₄), which makes it possible to build an efficient 800 nm trigged UCNP-based photoswitch without Nd³⁺-sensitized layer. If we go over the conventional UC switches based on Yb³⁺-Er³⁺ or Nd³⁺/Yb³⁺-Er³⁺ combination, the non-negligible UV-blue UC emission of Er³⁺ (e.g. ~410 nm and
~380 nm, see Figure 4.5) is a negative factor for real-time imaging in vivo (vide supra). This defect can be overcome in our structure where the UV-blue emissions (even the green emission) of Er$^{3+}$ are sufficiently quenched due to the robust Er$^{3+}$-Er$^{3+}$ cross-relaxation in the NaErF$_4$ core. As shown in Figure 4.5, compared with the traditional Nd$^{3+}$/Yb$^{3+}$ sensitized UC structure, the NaErF$_4$-based structure reduces the UV-blue emission by at least one order of magnitude while the red emission remains effective. Therefore, our design guarantees a better control of UV-blue emission, which is in favor of photoswitching involved applications (e.g. real-time UCL imaging and imaging-guided treatment).

Figure 4.5 Upconversion emission spectra of (a) NaErF$_4$@NaYF$_4$ and NaYF$_4$: 20% Yb, 2% Er@20%Yb@20%Nd nanoparticles under 800 nm excitation (b) NaErF$_4$@NaYF$_4$ and NaYF$_4$:20%Yb, 2%Er@NaYF$_4$ nanoparticles under 980 nm excitation in cyclohexane solution. The excitation power densities are 30 W cm$^{-2}$. All the spectra are normalized by the ~660 nm red emission.

Next, we optimize the structure of the PUCNPs. As far as the luminescence is concerned, the structure can be divided into two parts, namely, the NaErF$_4$@NaYF$_4$ core-S1 part and NaYbF$_4$: 0.5% Tm@NaYF$_4$ S2-S3 part. For the first part, the S1 shell thickness is important. As shown in Figure 4.4c, the UC luminescence of this part is significantly increased when the NaYF$_4$ shell gets thicker, until ~4.5 nm, which is in line with our previous report.$^{25}$ Besides, thicker S1 layer blocks better the energy transfer between the luminescent core (NaErF$_4$) and S2 layer (NaYbF$_4$: 0.5% Tm), in favor of strong UC luminescence. As shown in Figure 4.6, if S1 layer is not thick enough, say ~3 nm in our case, the components of the two parts will be diffused to a certain extent to the S1 layer which is unavoidable during the high temperature synthesis, and the diffused ions may interact with each other in the S1 layer which shall significantly reduce the UC emission of S2 layer (the UC emission of Tm$^{3+}$ can be efficiently quenched by the diffused Er$^{3+}$). This argument is further supported by the quenching effect of Nd$^{3+}$ in our designed NaErF$_4$@NaYF$_4$@NaYF$_4$: 80%Nd sandwich nanostructure (see Figure 4.7). It is found out that only when the barrier layer is thicker than ~4.5 nm, the quenching effect of Nd$^{3+}$ on the NaErF$_4$ core could be completely ignored, in agreement with results of Figure 4.6. Therefore, in the structure studied, the S1 layer thickness is set as ~4.5 nm.
Figure 4.6 The upconversion emission of PUCNPs with S1 (NaYF₄) layer (3 nm) nm (black line) and 4.5 nm (blue line) upon 980 nm laser excitation, respectively.

Figure 4.7 TEM images of (a) NaErF₄@NaYF₄ (3 nm)@NaYF₄:80%Nd (3 nm) (Er@Y@80%Nd), (b) NaErF₄@NaYF₄ (4.5 nm)@NaYF₄:80%Nd (3 nm). The Nd³⁺ diffusion effect diffusion effect study by comparing the emission spectra of (c) Er@Y(3 nm)@80%Nd(3 nm) and NaErF₄@ NaYF₄ (6 nm), (d) Er@Y(3 nm)@80%Nd(3 nm) and NaErF₄@ NaYF₄:10% Nd (6 nm), (e) Er@Y(4.5 nm)@80%Nd(3 nm) and NaErF₄@ NaYF₄ (4.5 nm)@ NaYF₄ (3 nm). All the UCNPs have the same size but different shell thickness.

As to the second part, high doping concentration of Yb³⁺ is used to achieve a robust UV-blue emission, which is rationalized by a simple sandwich structure: NaYF₄@NaYF₄: x% Yb 0.5% Tm@NaYF₄ (x: 20-99.5). With the similar sizes (38±3 nm) and morphologies of as-synthesized UCNPs (confirmed by the SEM images in Figure 4.8), the UC emission is enhanced significantly with the increase of Yb³⁺ concentration, as shown in Figure 4.4d. Compared to the most popular doping concentration (i.e. 20%), Yb³⁺ concentration of 99.5% results in 40 times enhancement
(emission intensity integrated range from 250 nm to 500 nm). This effect is ascribed to “size confinement effect” in the specific inert core-active shell-inner shell nanostructure. Based on above results and discussion, the PUCNPs is optimized as NaErF$_4$ (~15 nm)@NaYF$_4$ (~4.5 nm) @NaYbF$_4$: 0.5%Tm (~3.5 nm) @NaYF$_4$ (~4.5 nm).

**Figure 4.8** SEM images of the as-synthesized NaYF$_4$@NaYF$_4$: x% Yb, 0.5%Tm @NaYF$_4$ core-shell-shell nanoparticles with the changing concentration of Yb$^{3+}$ from 20 to 99.5%. All the scale bars are 200 nm.

### 4.2.3 Biological applications of PUCNPs

These PUCNPs are feasible for the application in theranostics. We demonstrate here, as a proof-of-concept, the features of separately controlling the upconversion luminescence (UCL) imaging (diagnosis) and photodynamic therapy (PDT) of cancer using PUCNPs@TiO$_2$ nanophotosensitizers (NanoPS). Considering that the photocatalyst-titanium dioxide (TiO$_2$) can only absorb UV light to catalyze the generation of reactive oxygen species (ROS), the PUCNPs@TiO$_2$ NanoPS can induce PDT only by 980 nm excitation (as shown in Figure 4.9c).

Here we coated a layer of TiO$_2$ nanoparticles on the surface of the PUCNPs with the help of polyvinylpyrrolidone (short as PVP, average mol wt 40 000). The synthesis and photoswitching function of NanoPS are illustrated in Figure 4.9a. 800 nm laser excitation only generates quasi-monochromatic red emission for UCL imaging as diagnosis. Once the tumor site is targeted, the excitation shall switch to 980 nm to induce additional UV emission for PDT. Therefore, the
photoswitching manner of the designed NanoPS guarantees the safety of imaging and the precision of therapy. The TEM image in Figure 4.9b reveals that some TiO\textsubscript{2} nanoparticles are decorated on the surface of PUCNPs as antennas, indicating the successful preparation of the PUCNPs@TiO\textsubscript{2} nanoplatform. Furthermore, the selective energy transfer efficiency between PUCNPs and TiO\textsubscript{2} is identified to 63\%, as calculated from the dropped UV/red emission ratio subject to the TiO\textsubscript{2} modification (Figure 4.9c). The experimental details involved above can be referred to Section 4.4.

![Scheme of photoswitchable PDT using PUCNPs@TiO\textsubscript{2} for real-time monitoring and image-guided phototherapy](image)

**Figure 4.9** (a) Scheme of photoswitchable PDT using PUCNPs@TiO\textsubscript{2} for real-time monitoring and image-guided phototherapy. (b) TEM image of PUCNP@TiO\textsubscript{2} nanoparticles. (c) Upconversion emission spectra of PUCNPs@ligand-free (red line) and PUCNPs@TiO\textsubscript{2} (blue line), the corresponding absorbance of PUCNPs@TiO\textsubscript{2} is given as the dotted line.

Before the application of PUCNPs@TiO\textsubscript{2} in vivo, its dark cytotoxicity was evaluated in vitro using A549 cells (Human lung adenocarcinoma epithelial). As shown in Figure 4.10, cell viability is higher than 85\% even with a relatively high concentration (800 µg/mL) of PUCNPs@TiO\textsubscript{2}. To study the cell imaging profiles, A549 cells were incubated with the PUCNPs@TiO\textsubscript{2} at 200 µg/mL overnight and the cell nuclei were stained by DAPI, giving the blue emission as shown in Figure 4.13a. According to the overlapped channels, the UCL of PUCNPs@TiO\textsubscript{2} appears around the nucleus, indicating the cellular internalization of the nanoplatform. Comparison between the UCL blue and red channels under different laser excitations (980 nm and 800 nm, respectively) comes out that only 980 nm excitation induces a bright blue UCL, whereas the red UCL can be generated by both 980 nm and 800 nm excitation.
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Figure 4.10 Cell viabilities of A549 cells treated with PUCNPs@TiO$_2$. Error bars indicate standard deviations, n = 3.

Subsequently, the ROS generation of PUCNPs@TiO$_2$ nanoplatform was studied, where fluoresceinyl cypridina luciferin analogue (FCLA) was used as a specific chemiluminescence probe of $^{1}$O$_2$ and O$_2^-$. As shown in Figure 4.11, 980 nm excitation of the nanoplatform results in a cumulative chemiluminescence — a symbol of the increase of ROS generation. On the contrary, 800 nm light irradiation has no such effect. The corresponding standard curves are given as well to reconfirm it. Importantly, the intracellular ROS generation was also confirmed by the non-fluorescent DCFH-DA probe, which could be deacetylated and further oxidized by ROS to the fluorescent DCF. As shown in Figure 4.12, compared to the control group and 800 nm laser treated group, the 980 nm laser treated group is proved to have effective generation of ROS, showing the strong fluorescence. The results above confirm the mechanism of ROS-mediated photoswitchable PDT.

Figure 4.11 Chemiluminescence intensity of FCLA treated with PUCNPs@TiO$_2$ under (a) 980 nm and (b) 800 nm laser irradiation at different time, respectively (power density: 0.5W/cm$^2$). Standard curve of ROS generation with the increase of PUCNPs@TiO$_2$ concentration under(c) 980 nm laser and (d) 800 nm laser irradiation at 0.5W/cm$^2$ power density for 5 min.
Following the confirmation of ROS generation, the PDT effect of PUCNPs@TiO$_2$ against A549 cancer cells was evaluated by MTT assay. Dark toxicity, as a control, was also evaluated. After 24 h cell incubation, the difference in cell viability is clear when switching the wavelengths of irradiation. Upon 980 nm laser irradiation at 1 W/cm$^2$, the cell viability (as shown in Figure 4.13b) decreases significantly, which is more profound with higher ROS dosage. For example, at 400 µg/mL concentration, cell viability is down to 15%, indicating a robust therapeutic effect. But there is no treatment effect under 800 nm laser excitation with the same light dosage.

On the basis of the *in vitro* results, the *in vivo* UCL imaging and photoswitchable PDT were carried out. For UCL imaging, intravenous (IV) injection was conducted on tumor-bearing mice with of 100 µL of PBS or PBS containing PUCNPs@TiO$_2$ (10 mg/mL). At 2 h and 24 h after the injection, the tumor-bearing mice were exposed to the non-therapeutic 800 nm laser, as shown in Figure 4.13c. Compared to the control group, after 2 h the mice injected with the as-designed nanoplatform has already exhibited strong UCL in the tumor site. 24 h later, the UCL intensity is observed to increase in the same area. Our results suggest the ability of PUCNPs@TiO$_2$ to accumulate in tumor site with the time increasing owing to the enhanced permeability and retention (EPR) effect. Then all the tumors and major organs of these mice were taken out for the *ex vivo* UCL imaging (Figure 4.13d). The tumor of the mice treated with PUCNPs@TiO$_2$ shows a strong luminescence signal. The signals are also observed in liver, spleen and kidney, while negligible signals are observable in other organs such as heart, lung (not provided). The results can qualitatively reflect the distribution of PUCNPs@TiO$_2$ in the body after IV injection.
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Figure 4.13 (a) A549 cells treated with PUCNPs@TiO$_2$ for imaging with 980 nm and 800 nm light excitation, respectively, and stained with DAPI. The scale bars are 20 µm. (b) A549 cells treated with PUCNPs@TiO$_2$ upon 800 nm and 980 nm laser irradiation with the same power densities. (c) In vivo UCL imaging of LLC tumor-bearing mouse with PUCNPs@TiO$_2$ under 800 nm laser at 2 h and 24 h after intravenous injection. (d) Ex vivo UCL images of kidney, spleen, liver and tumor after 24 h post injection, the luminescent intensity of dissected organs further confirmed the preferential accumulation of PUCNPs@TiO$_2$ in tumor and main organs. (e) Photographs to display the excised tumors after various treatment. (f) H&E staining tumor sections harvested from the control group, groups of PUCNPs@TiO$_2$ only and PUCNPs@TiO$_2$ + 800 nm and PUCNPs@TiO$_2$ + 980 nm laser. The scale bars are 50 µm. (g) Relative tumor volumes change during the treatment (n=5).

To evaluated the photoswitchable PDT effect, Lewis lung carcinoma (LLC) tumor-bearing C57/6J mice were intravenously injected with PUCNPs@TiO$_2$, and the tumor part was irradiated by 980 nm and 800 nm laser with the same light dosage at 0.5 W/cm$^2$ for 15 min irradiation (3 min irradiation each time with 3 min interval, 5 cycles), respectively. In addition, the group injected with UCNPs@TiO$_2$ without laser irradiation, the group only irradiated by 980 nm or 800 nm laser, as well as the group without any processing, were set as control groups. As shown in
Figure 4.13e, the excised tumors from these groups have confirmed the inhibition effect—the tumor size under 980 nm triggered PDT treatment is the smallest, whereas there is no significant difference among the other groups, even for the 800 nm triggered PDT treatment group with the same light dosage, which features the advantage of the as-designed photoswitchable PUCNPs@TiO$_2$ NanoPS in separating diagnosis (800 nm laser excitation) and photodynamic therapy (980 nm laser excitation).

To further confirm the therapeutic effect, the histological changes of corresponding excised tumors were evaluated by using hematoxylin and eosin (H&E) staining in Figure 4.13f. There are more significant damages in tumor cells with the injection of PUCNPs-TiO$_2$ (with 980 nm irradiation) than those in control groups. The histological analysis was also performed in heart, liver, spleen, lung and kidney (as shown in Figure 4.14) and no pathological changes were observed between the untreated group and 980 nm triggered PDT treatment group, indicating the harmlessness of the PUCNPs-TiO$_2$ NanoPS to normal organs. According to the overall therapeutic effect as shown in Figure 4.13g, only the group received the injection of PUCNPs@TiO$_2$ and 980 nm irradiation exhibits a significant PDT effect as evidenced by the lowest tumor growth rate. By contrast, the tumors of the other control groups grow continuously under the same conditions. Thus, the as-designed PUCNPs can be a candidate for NIR light-triggered photoswitchable nanoplatform with potential application in precise medicine.

**Figure 4.14** Representative histological H&E staining of various organ tissues collected from different groups. All the scale bars are 50 µm.

### 4.3 Conclusion

A Nd$^{3+}$ free photoswitchable upconversion nanomaterial, i.e. NaErF$_4$@NaYF$_4$@NaYbF$_4$:0.5%Tm@NaYF$_4$ is realized. This structure succeeds in a complete photoswitching in UV-blue region, compared to the current Nd$^{3+}$ photoswitching materials. The features of this nanostructure are (i) the core is dopant free and thus a prominent quasi-monochromatic red upconversion emission of Er$^{3+}$ is realized, (ii) the intermediate layer thickness is set to 4.5 nm to avoid diffusion effect and (iii) the doping concentration of Yb$^{3+}$ ions is raised to 99.5% to simplify the sandwich structure and achieve the robust UV-blue upconversion emission. As a proof of concept, we have constructed PUCNPs@TiO$_2$ as photoswitchable nanophosensitizers to demonstrate its ability of imaging-guided "off-on" therapy. Our results indicate that this
photoswitchable upconversion nanostructure can be expected to have promising applications in biomedicine.

4.4 Experimental details

**Reagents**: RECl$_3$·6H$_2$O (Re: Y, Yb, Er, Nd, Tm >99%), oleic acid (OA, 90%), 1-octadecene (ODE, 90%), oleylamine (70%), CF$_3$COONa, (CF$_3$COO)$_3$Y, and TiF$_4$ were purchased from Sigma-Aldrich. RE$_2$O$_3$ (Re: Tm, Yb) was purchased from Aladdin. NaOH, NH$_4$F, methanol, ethanol and cyclohexane were purchased from GFS Chemical. The immunol staining fix solution and 4’,6-Diamidino-2-Phenylindole (DAPI) were purchased from Beyotime Biotechnology. Fluoresceinyl cypridina luciferin analogue (FCLA) was obtained from Tokyo Kasei Kogyo Co., Tokyo, Japan. All the chemicals were of analytical grade and there was no further purification. The cell lines used in this study were obtained from Basic Medical Sciences, Jilin University.

**The preparation of PUCNPs**: In order to synthesis the multi-shelled NaErF$_4$@NaYF$_4$@NaYbF$_4$: 0.5% Tm@NaYF$_4$ nanoparticle, an epitaxial layer-by-layer growth Ostwald ripening method was employed.$^{27}$ The preparation of precursors of each shell prepared in advance. The synthesis of bare core (NaErF$_4$) was following a previously reported procedure with some modification.$^{27-28}$

**Synthesis of bare core NaErF$_4$ nanoparticles**: In a typical experiment, 1 mmol ErCl$_3$ was added in a 50 mL three-neck flask with 6 mL OA and 15 mL ODE. The mixture was stirred at 160 °C for 30 min under an argon atmosphere until a clear light-pink solution was obtained. Then it was cooled down to room temperature. After being added with 5 mL methanol solution containing 2.5 mmol NaOH and 4 mmol NH$_4$F, the clear solution turned into opacity and was heated to 70 °C in air for 20 min to remove methanol molecules. With argon gas protection, the solution was heated to 300 °C (with a rate of 10°C per minute) and maintained for another 90 min with stirring. The resulting nanoparticles were first washed with acetone and then washed with ethanol twice. Then they were dispersed in 4 mL cyclohexane.

**NaYF$_4$ precursor**: Typically, 2 mmol CF$_3$COONa and 2 mmol (CF$_3$COO)$_3$Y were added into a 100 mL flask with 6 mL OA, 10 mL ODE and 6 mL oleylamine. The mixture was robustly stirred at 160 °C for 30 min to dissolve the reagents. Then the solution was directly heated to 290 °C for 1 hour and cooled down to room temperature. The obtained precursor was precipitated by adding excess ethanol. Then after centrifugation, the products were finally re-dispersed in 4 mL ODE.

**NaYb(x)F$_4$: Tm (0.5% mol) precursor**: A set of NaYb$_x$F$_{4-x}$: 0.5% Tm precursor was prepared with increasing concentrations of Yb$^{3+}$ sensitizers (20%, 40%, 60%, 80% and 99.5%). Briefly, Tm$_2$O$_3$ (1 mmol) was dissolved in 50% aqueous trifluoroacetic acid (20 mL), refluxed overnight at 95 °C, and dried overnight at 70 °C remaining ~2 mmol Thulium trifluoroacetate as white powder. Similarly, the Ytterbium trifluoroacetate was obtained. The Tm$^{3+}$ concentrations were fixed at 0.5% to ensure a uniform Tm$^{3+}$ content in these samples. Taking NaYbF$_4$: 0.5%Tm precursor as an example. 1.99 mmol (CF$_3$COO)$_3$Yb, 0.01 mmol (CF$_3$COO)$_3$Tm, 2 mmol CF$_3$COONa were added into a 100 mL flask with 6 mL OA, 10 mL ODE and 6 mL oleylamine. The following preparation was conducted in a similar procedure.

**General procedure for the synthesis of core-shell-shell nanoparticles (NaYF$_4$@NaYb (x) Y (99.5%-x) F$_4$: 0.5% Tm@NaYF$_4$):** The synthesis of NaYF$_4$ core was prepared as the mentioned procedure of NaErF$_4$. The as-obtained NaYF$_4$ nanoparticles are dissolved in 11 mL cyclohexane solution for further use. Briefly, 2 mL cyclohexane solution containing 0.5 mmol NaYF$_4$ bare core
nanoparticles were mixed in the 100 ml three-neck flask with 6 ml OA, 15 ml ODE. Protected by argon gas, the solution was rapidly heated to 300 °C. When the solution temperature reached 300 °C, 1 mmol NaYb(x)Y(99.5%-x) F₄: 0.5% Tm precursor and 2 mmol NaYF₄ precursor were injected into the solution in sequence, each with a reaction time of 30 min to form corresponding epitaxial shells. After the entire reaction, the mixture was cooled down and the resultant products were collected by centrifugation with acetone once and ethanol twice.

**Synthesis of multi-shelled nanoparticles** (NaErF₄@NaYF₄@NaYbF₄: 0.5%Tm@NaYF₄): The procedure is similar to core-shell-shell nanoparticles and just take core-shell of NaErF₄@NaYF₄ as NaYF₄ bare core. The PUCNPs with two-way of excitation was optimized by varying the doping content to increase shell thickness. According to the results above, NaErF₄@NaYF₄@NaYbF₄: 0.5%Tm@NaYF₄ with various shell thicknesses were synthetized.

**Synthesis of NaErF₄@NaYF₄@NaYF₄: Nd nanoparticles:** The procedure is similar to the above protocol with different thickness of NaYF₄ layer.

**Preparation of PUCNPs@TiO₂ nanoplatform for PDT:** The PUCNPs@TiO₂ nanoplatform was prepared according to the reported method.³⁰⁻²⁹ First, the water soluble ligand-free of PUCNPs was obtained by removing the surface ligand (oleic acid, OA) on the nanoparticles. Typically, 4 mL 0.1 M HCl solution was added to the 4 mL of PUCNPs-OA cyclohexane solution. And the mixture was stirred vigorously until the PUCNPs were transferred to the water phase, monitored by the 980 nm laser. The lasers used in this work were MDL-980 nm (fiber coupled, 1mW-10W), MDL-808 nm (fiber coupled, 1mW-10W) from Changchun New Industries Optoelectronics Technology Co., Ltd. The PUCNPs were separated from the below solution and centrifuged at 12000 rpm for 15 min. After that, the supernatant was discarded and the ligand-free nanoparticles were purified by washing with di-water for three times and finally re-dispersed for further use. The concentration of PUCNPs was determined by weighing out the dried nanoparticles in solution.

**Detection of reactive oxygen species (ROS):** ROS was detected by FCLA according to a previously published method.³⁰ First of all, to confirm the photoswitch manner of ROS generation, the time-dependent ROS production at the same concentration of PUCNPs@TiO₂ was studied. Briefly, 1 mL of the reaction mixture contained PUCNPs@TiO₂ (400 μg/mL), 0.06 μg/mL of FCLA (prepared just before use) and 0.01 M PBS (pH 7.4) in a standard quartz cuvette (1×1×4 cm). Then the mixture was irradiated by 800 nm and 980 nm lasers light for (0, 5, 10, 15 min) at the same photodensity of 0.5W/cm². After separated from the PUCNPs@TiO₂ nanoparticles by centrifugation, the supernatant was immediately measured by fluorescence spectrometer, respectively. Then, the concentration-dependent ROS production was also studied. Similar experimental methods were performed. The concentration of ROS produced by PUCNPs@TiO₂ (a serial of concentration) under 980 nm or 800 nm excitation at 0.5W/cm². To guarantee that there is enough dissolved oxygen, we only irradiated for 5 min with stirring.
The generation of ROS was also confirmed by the intracellular experiment using DCFH-DA probe. Typically, A549 cells were seeded on the glass bottom of cell-culture plates at a density of $1.5 \times 10^4$ cells/well. After that, the cells were incubated with PUCNPs@TiO$_2$ (200 µg/mL) at 37 °C for 12 h and then treated with DCFH-DA (10 µM) at 37 °C for 20 min. The culture medium was discharged and the cells were rinsed with medium. Finally, the cells were treated with 800 nm and 980 nm laser at 0.5 W/cm$^2$ power density for 10 min, respectively. The fluorescence intensity of the cells was evaluated by confocal laser scanning microscopy (CLSM) (Ex= 488 nm and Em= 500-540 nm).

### 800 nm for real-time Cellular imaging and 980 nm for PDT with photoswitchable smart PUCNPs nanoprotocol in vitro:

Based on the smart multifunctional two-way of NIR photoswitch PUCNPs. As a proof of concept, we performed the real-time image-guided PDT in A549 cells. Briefly, the cellular uptake profiles and irradiation wavelength dependent cytotoxicity was evaluated.

For cellular imaging, A549 cells were seeded on the 96-well cell-culture plates ($1.5 \times 10^4$ cells/well) for 24 h with Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS). Then the medium was replaced by the fresh medium containing PUCNPs@TiO$_2$ nanoprotocol, followed by another 12 h incubation. Afterward, the cells were rinsed with PBS, fixed with immunostaining fix solution and stained with DAPI for 5 min. Then it was examined by a modified CLSM with an upconversion system (Nikon microscope equipped with 980 nm and 800 nm lasers) upon different excitation wavelengths.

For PDT study, A549 cells were seeded in 96-well plates at a density of $1 \times 10^4$ cells per well. After confluent growth for 24 h, the culture medium was discharged and incubated with fresh medium containing various concentration of PUCNPs@TiO$_2$. A standard MTT method was explored to examine the cytotoxicity of as-prepared PUCNPs@TiO$_2$ after another 24 h incubation. On the basis of dark cytotoxicity results, the in vitro PDT was carried out. The same concentration of PUCNPs@TiO$_2$ and the same irradiation power densities (1W/cm$^2$) under 800 nm and 980 nm light were exposed to the predetermined concentration of cell wells for 15 min, respectively. Similarly, the cell viability was evaluated by MTT assay.

### Photoswitching PDT and imaging in vivo:

All the animal studies were approved in conformity with the animal management protocols. The tumor-bearing mice were successfully obtained by the subcutaneous injection of mouse Lewis lung carcinoma (LLC) cells into C57 mice. When the tumors were grown to a size of around 50 mm$^3$. The tumor-bearing mice were randomly assigned into six different groups: (i) untreated control, (ii) control (980 nm NIR-light alone), (iii) control (800 nm NIR-light alone) (iv) PUCNPs@TiO$_2$ alone, (v) PUCNPs@TiO$_2$ with 800 nm laser light and (vi) PUCNPs@TiO$_2$ with 980 nm PDT, and each group contained of 5 mice. Except for the control groups, all the other groups were intravenously injected with 100 µL of PUCNPs@TiO$_2$ (10 mg/mL) but with differently treated strategies. Tumor size was measured after treated for 2 weeks. The calculation of tumor volume was conducted by applying the following formula: $\text{Volume} = (ab^2/2)$, where $a$, and $b$ are tumor dimensions in 2 orthogonal directions. All the animals were sacrificed at day 14. Xenografts were dissected free of surrounding tissue and fixed with 4% paraformaldehyde. In vivo UCL imaging was performed with an IVIS spectrum, modified with the equipment of an 800 nm CW laser as the excitation source, in a similar way as described in a previous paper.\textsuperscript{31}
References


