Photoswitchable upconversion nanoparticles with excitation-dependent emission for programmed stepwise NIR phototherapy

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Photoswitchable upconversion nanoparticles with excitation-dependent emission for programmed stepwise NIR phototherapy

Highlights
An energy segmentation-based strategy is proposed to develop photoswitchable UCNPs
The developed UCNPs enable controlled activation of imaging, gas therapy, and PDT
Tumor hypoxia issue can be alleviated, showing its strong therapeutic promise

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**SUMMARY**

Programmable control over therapeutic processes in phototherapy, like photodynamic therapy (PDT), is promising but challenging. This study uses an energy segmentation-based strategy to synthesize core-multi-shell upconversion nanoparticles (UCNPs), which can release three different colors (red, green, and blue) upon exposure to different near-infrared light (1550 nm, 808 nm, and 980 nm). By combining these UCNPs with photosensitizers and nitric oxide (NO) donors, a smart “off-on” PDT nanoplatform is developed. UCNPs enable independent activation of imaging, release of NO, and generation of reactive oxygen species using specific light wavelengths. The results show that sequential NO release before PDT can greatly alleviate tumor hypoxia by reducing oxygen consumption. This stepwise approach shows potential for precise NIR light-activated and imaging-guided phototherapy.

**INTRODUCTION**

Photodynamic therapy (PDT) is a type of light therapy that is widely used to treat various malignant diseases with minimal invasiveness and toxicity. Typically, three elements are involved in a PDT process: excitation light, photosensitizing molecules (photosensitizers), and oxygen. Upon irradiation with light of an appropriate wavelength, the photosensitizer may undergo a transition from an excited singlet state to a triplet state, which may then react with nearby oxygen (O₂) and produce singlet molecular oxygen. As the main reactive oxygen species (ROS), these highly cytotoxic singlet oxygen molecules can cause irreversible cellular damage and induce cell death or tumor destruction. Despite its great achievements, PDT still faces several challenges. Firstly, commonly used photosensitizers are mainly activated by ultraviolet or visible light with limited tissue penetration depth, which in turn restricts the application of PDT to superficial lesions. Secondly, as an oxygen-dependent therapy, the therapeutic efficiency of PDT largely depends on the presence of ambient oxygen. For example, the yield of singlet oxygen of aloe emodin (AE) photosensitizers may drop to nearly zero when oxygen is absent. Unfortunately, the tumor microenvironment is usually in a hypoxic state due to unbalanced oxygen supply. Furthermore, damage to the vasculature and continuous depletion of oxygen during PDT may even further exacerbate hypoxia. Therefore, hypoxia is considered as a causative factor for tumor resistance to PDT and many other therapeutic strategies including chemotherapy, radiotherapy, etc.

Lanthanide-ion doped upconversion nanoparticles (UCNPs) can convert long-wavelength excitation light (e.g., NIR) to short-wavelength emission light (UV or visible). Since NIR light has a deeper tissue penetration depth than UV and visible light due to the minimal absorbance of biomolecules in the NIR spectral window (700–1000 nm), UCNPs can be used as light transducer to excite photosensitizers in deep tissues. Various photosensitizers, e.g., zinc phthalocyanine (ZnPc), merocyanine 540 (MC540), chlorin e6 (Ce6), rose bengal, curcumin, TiO₂, and black phosphorus nanosheets (BPNS), have been combined with UCNPs and demonstrated great potentials for deep-tissue PDT applications. In addition to improving the tissue penetration of light, many efforts have been devoted to overcoming other obstacles in PDT, namely hypoxia and on-demand activation. For example, MnO₂ layer and Fe(OH)₃ compounds have been used in UCNP-based PDT because these nanocatalysts can regulate hypoxic tumor microenvironment by decomposing intracellular hydrogen peroxide (H₂O₂). Erythrocyte-sized hemoglobin microgels have also been combined with UCNPs because they can generate oxygen, heat and ROS to suppress hypoxic tumor. In other studies, orthogonal emissive UCNPs have been used for imaging-guided PDT treatment, where one emission is used to trigger the release of ROS while the other is used for diagnostic purpose.

Ideally, the PDT process should be properly controlled and optimized to deliver the photosensitizer to the lesion, subsequently suppress tumor hypoxia, and then release ROS in a step-by-step manner. In other words, imaging guidance, hypoxia suppression and ROS release...
should be performed or triggered in sequence, giving the right treatment at the right time and for the right duration. However, such programmable PDT requires independent and sequential photoactivation of different therapeutic processes using different excitation lights, which cannot be achieved using conventional UCNPs excitable at a single wavelength only. In this study, an energy segmentation-based strategy is proposed to synthesize core-shell UCNPs (NaYF₄@NaErF₄:Tm@NaYF₄@NaYF₄:Yb,Ho,Nd@NaYF₄@NaYF₄:Yb,Tm) with excitation wavelength-dependent emission, i.e., red emission when excited at 1550 nm, green emission when excited at 808 nm, and blue emission when excited at 980 nm. By carefully tuning the thickness of the luminescent shell and inert shell, the excitation and emission of the three luminescent ions are strictly segmented in different shell regions. Therefore, unwanted crosstalk between these ions can be efficiently avoided and high color purity can be obtained. The specially designed UCNPs are loaded with the photosensitizer zinc phthalocyanine (ZnPc) and nitric oxide (NO) donor Roussin’s black salt (RBS) (Scheme 1). Under 980 nm excitation, the blue emission of UCNPs can excite the RBS salt to produce NO gas. The released NO will inhibit cellular respiration by competing with oxygen for binding to mitochondria so less oxygen is consumed. When the excitation wavelength is switched to 1550 nm, ZnPc is then activated by the red emission of UCNPs. Since tumor oxygenation is greatly increased by inhibiting mitochondrial respiration, the tumor hypoxic environment is relieved and more ROS can be generated. The green emission of UCNPs under 808 nm excitation is used for imaging guidance and identification of lesion areas, as no ROS or NO is generated.

It is important to note that the release of NO and ROS must be activated in a cascaded manner. If they are activated simultaneously, there is not enough time to inhibit cellular respiration and PDT will still suffer from hypoxia. Our results demonstrate that precise control of the PDT process and stepwise activation of NO release and ROS production can significantly reduce cancer cell viability and limit tumor growth.

RESULTS

The structure of as-developed photoswitchable UCNPs, NaYF₄@NaErF₄:Tm@NaYF₄@NaYF₄:Yb,Ho,Nd@NaYF₄@NaYF₄:Yb,Tm (C@S1@S2@S3@S4@S5), can be divided into three luminescent and three inert regions. As shown in Figure 1A, NaErF₄:0.5%Tm (S1) layer...
can be activated under 1550 nm excitation and release red emission, while NaYF₄:20%Yb,2%Ho,10%Nd (S3) shell emits green signal in response to 808 nm excitation and NaYF₄:Yb,0.5%Tm (S5) layer releases blue light under 980 nm excitation. Although the NaYF₄ shell is optically inactive, it is mainly used to enhance emission intensity and block energy transfer processes between different luminescent layers. As for the structure arrangement, since Er³⁺ ions can sensitize 808 nm, 980 nm, and 1550 nm excitation light (Supporting information Figure S1A), the Er³⁺ doped layer is particularly placed in the inner section of the whole core-multi-shell nanostructure. Figure S1B demonstrates a scenario where S1 and S5 shell are replaced with each other, i.e., NaErF₄ shell in the outermost region and NaYF₄:Yb, Tm layer in the first shell. In this case, the NaErF₄ shell in the outermost region would always be the first to be excited, resulting in the presence of red emission regardless of the excitation light used (Figure S1A). This would significantly affect the color purity. However, in Figure S1C, we arranged the shells differently, placing NaErF₄ in the S1 shell, NaYF₄:Yb,Nd,Ho in the S3 shell, and NaYF₄:Yb,Tm in the S5 shell. This is because the green luminescent layer, NaYF₄:Yb,Ho,Nd (S3), or blue luminescent layer, NaYF₄:Yb,Tm (SS), can function as a filter that, respectively, prevents 808 nm and 980 nm light from accessing to S1 layer. Under this circumstance, Er³⁺ ions in S1 shell can only respond to 1550 nm light and release red emission with high color purity. Besides, the Tm³⁺ dopant ion can further promote the red color emission of Er³⁺ ions via back-energy transfer between 3H₅ state of Tm³⁺ and 4H₁₃/₂ states of Er³⁺. As the pair of Nd³⁺-Yb³⁺ ions in the green emitting layer (S3) can respond to both 808 nm and 980 nm light, the S3 shell is then placed in the middle part of the whole nanostructure such that the incident 980 nm light can be blocked by the outermost blue emitting layer (S5). In the S3 shell, Nd³⁺ ions can sensitize 808 nm light and transfer the excitation energy to neighboring Yb³⁺ ions, then to Ho³⁺ activator ions for green emission. Moreover, the thickness of the S3 shell can be varied to regulate the amount of 808 nm photons reaching the S1 shell and control the color output. For the S5 part, the Yb³⁺ sensitizer absorbs 980 nm photons and transfer the energy to Tm³⁺ activators for the release of blue upconversion emission. Similar to S3, the S5 layer is also used as the 980 nm photon filter because its thickness can be tuned to confine migration of excitation energy within the S5 shell. By carefully optimizing the thickness of luminescent and inert shell, energy migration pathways upon different excitation lights (1550 nm, 980 nm, and 808 nm) can be precisely segmented and confined in the respective shell, which greatly favors the orthogonal red-green-blue upconversion luminescence (Figure 1B).

Figures 2A–2F present transmission electron microscopy (TEM) images of core and core-multi-shell nanoparticles, which reveal that as-synthesized UCNPs exhibit uniform size and shape. The lattice spacing of the core-quintuple-shell UCNPs is calculated to be 0.51 nm that corresponds to the (100) crystal plane of the hexagonal phase (Figure 2G). Figure S2 presents the size distribution of these particles with different shell thickness. The X-ray diffraction (XRD) also confirms that Core, Core@S1, Core@S1@S2, Core@S1@S2@S3, Core@S1@S2@S3@S4, and Core@S1@S2@S3@S4@S5, nanoparticles all exhibit hexagonal phase [Figure S3]. The Scherrer equation, represented as follows:

![Figure 1. Energy migration pathways and upconversion luminescence spectra of UCNPs under various light excitations](image)
is commonly used to evaluate the relationship between crystalline domain size \( D \), broadening of a peak at a particular angle \( q \), and the width of the peak at half of its height \( b \). The particle size increases with continuous coating of different shells. Consequently, it is expected that the half full bandwidth in the XRD peaks is expected to become narrower, which is not observed in Figure S3. There are several reasons for this inconsistency. Firstly, it should be noted that particle size does not necessarily correspond to the crystalline size, as particles may contain multiple crystalline domains. Additionally, the degree of crystallinity is reduced after several layers are deposited on the core, which may cause the broadening of the diffraction peak.

Because the contrast of the HAADF-STEM (high angle annular dark field-scanning transmission electron microscopy) image is sensitive to the atomic number \( Z \) of chemical element, HAADF-STEM technique is further used to identify the morphology of core-quintuple-shell UCNPs. As shown in Figure 2H, bright and dark shells can be observed that corresponds to heavy and light elements present in the nanoparticle, indicating the formation of core-multi-shell nanostructure. This can also be verified by the variation of EDX (energy dispersive x-ray) line profile analysis across the whole nanoparticle (Figure 2). Notably, the nominal chemical composition of the particle is NaYF\(_4@NaErF\(_4@NaYF\(_4\))\) Tm@NaYF\(_4@NaYF\(_4\)) Yb,Ho,Nd@NaYF\(_4@NaYF\(_4\)) Yb,Tm. Therefore, if all the elements in the core or shell region were strictly confined, Er and Yb elements should only be localized in a relatively inner and outer region. However, it is clear that Er and Yb elements are detected almost throughout the whole particle, indicating that the cations are inter-diffused during the synthesis procedure. \(^{41}\)

As mentioned previously, the Core@S1@S2(NaYF\(_4@NaErF\(_4@NaYF\(_4\)) part can emit red luminescence in response to 1550 nm excitation. Specifically, as the thickness of S2 shell is increased from 3.3 nm to 5.7 nm (Figure 3A), the intensity is enhanced by 9.8 times (Figure 3A). This is because the increase of the S2 shell enhances the distance between luminescence center and surrounding quenchers, which reduces the energy transfer between them and accordingly increases the red upconversion luminescence. However, if the thickness of S2 shell is further increased to 7.9 nm, the luminescence intensity is reduced (Figure 3A). This reduction in intensity can be explained by the increased accumulation of internal quenchers from the interfaces between sequentially coated shells, as previously reported. \(^{42}\) It is believed that these internal quenchers act as non-radiative quenching channels, leading to a reduction in upconversion intensity when the shell thickness is too thick. On the successful tuning of the red emission, the green-emitting layer, NaYF\(_4@NaYF\(_4@NaYF\(_4\)) (S3) is subsequently coated, which can absorb the 808 nm excitation light and generate green upconversion luminescence. Notably, the pre-existing S1 shell (NaErF\(_4@Tm\)) can also sensitize 808 nm light and releases red luminescence, which affects spectral purity. As a result, a series of Core@S1@S2@S3@S4 samples with different
thickness of S3 shell (t = 7.4 nm, 8.7 nm and 14.1 nm) are synthesized (Figure 3B). Under excitation by 808 nm light, the Nd³⁺ in the S3 layer absorb the excitation light and transfer the energy to the Yb³⁺ ions and then Ho³⁺ activators, via the energy transfer pathway, i.e., 808 nm / Nd³⁺ / Yb³⁺ / Ho³⁺. The green emission arises from the transition of (5S₂, 5F₄) → (5I₈) of Ho³⁺ ions, which dominates the emission color of Ho³⁺ ions (Figure S4 A). However, the pre-existing S1 shell (NaErF₄:Tm) can also sensitize 808 nm light and releases red luminescence, which affects spectral purity. Therefore, as shown in Figure S4B, the green to red (G/R ratio) intensity ratio is only 0.7 upon 808 nm light excitation when the thickness of S3 is about 7.4 nm. As the thickness of S3 shell is increased, the G/R ratio value is accordingly increased. The green luminescence already dominates the upconversion emission when the thickness of S3 shell reaches 14.1 nm. This is because the S3 shell acts as a filter and it can prevent the 808 nm light from reaching the inner S1 shell. 

As for the blue-emitting portion, a series of Core@S1@S2@S3@S4@S5 nanoparticles with different thickness of S5 shell are investigated (Figure 3C). Upon excitation by 980 nm light, Yb³⁺ ions in the S5 shell absorb the excitation light and transfer the energy to Tm³⁺ activator ions. The major emission peaks at around 450 and 475 nm can be assigned to the 1D₂ → 3F₄, 1G₄ → 3H₆ transitions of Tm³⁺ ions (Figure S5 A). Notably, the 980 nm light can also be absorbed by the Yb³⁺-Ho³⁺ ions in the S3 shell, resulting in green emission. For example, the blue to green intensity ratio (B/R ratio) is only about 0.34 upon 980 nm light excitation when the thickness of S5 layer is only about 4.4 nm (Figure S5B). However, as the thickness of S5 shell is increased, the Yb³⁺-Tm³⁺ pair can act as a photon filter and significantly deplete the 980 nm light, inhibiting the inward propagation of incident light. As a result, by carefully controlling the thickness of the shell 5 layer, the dominant blue emission of Tm³⁺ ions can be acquired.

Next, we studied the emissive profiles of as-prepared core-multi-shell UCNPs at NIR lights with different power densities (Figures 3D–3F). It shows that the red, green, and blue emissions enhance gradually with increasing the excitation power of 1550 nm, 808 nm, and 980 nm light accordingly. Besides, the line fitting results indicate that there is a three-photon absorption process under 1550 nm and 980 nm excitation, and a two-photon absorption process under 808 nm excitation (Figure S6).

**Synthesis of mesoporous-silica-coated UCNPs**

The as-synthesized UCNPs usually contain hydrophobic ligands, such as oleic acid, which restrict their biological applications. In order to improve the water solubility and biocompatibility, mesoporous silica (mSiO₂) is coated on the surface of UCNPs (UCNPs@mSiO₂). As shown in Figure 4A, a uniform mesoporous layer (~5 nm thick) is decorated on the surface of UCNPs. According to the N₂ adsorption/desorption isotherm curve, the surface area of UCNPs@mSiO₂ is calculated to be 679 m² g⁻¹ and the average pore size is about 4.32 nm (Figure S7).
Construction of UCNPs@mSiO₂-ZnPc-RBS nanoplatform

Figure 4B shows the upconversion luminescence spectra of UCNPs@mSiO₂ under different NIR laser light excitations. Similar to the core UCNPs, UCNPs@mSiO₂ nanocomposite still enables to emit green, blue, and red luminescence under 808 nm, 980 nm, and 1550 nm laser light, respectively. Subsequently, two types of photosensitizer drugs, i.e., zinc phthalocyanine (ZnPc) and RBS salt were loaded into porous channels of UCNPs@mSiO₂ nanocomposite in order to construct a programmable therapeutic nanoplatform (UCNPs@mSiO₂-ZnPc-RBS). The reason why ZnPc photosensitizer is chosen as the 1O₂ generator for PDT treatment is because it has a strong absorption in the spectral region of 600–700 nm (the black dashed line in Figure 4B), which well matches the red light emission of UCNPs@mSiO₂ under 1550 nm excitation. Meanwhile, RBS salt is used as the NO donor because its absorption spectrum (the gray dashed line) overlaps with the ultraviolet emission (< 400 nm) of UCNPs@mSiO₂ under 980 nm excitation. As a result, the specially designed UCNPs@mSiO₂-ZnPc-RBS nanocomposite can independently release NO and ROS in response to two different excitation lights, which ensures the occurrence of different photoactivations of therapeutic at the right place and right time.

Independent release of ROS and NO in solution

First, 1,3-diphenylisobenzofuran (DPBF) dye was used as a probe to detect the generation of ROS because its absorbance at around 417 nm could be specifically decreased by ROS. Figure 4C shows the absorbance changes of DPBF after 30 min of NIR light under different conditions. It can be observed that the absorption peak of DPBF drops rapidly only under 1550 nm light excitation while no significant absorbance changes of DPBF dye under either 980 nm or 808 nm light excitations, indicating that ROS signals are mainly produced by UCNPs@mSiO₂-ZnPc-RBS nanocomposites under 1550 nm light excitation. Griess analysis kit method was then used to probe the production of NO of UCNPs@mSiO₂-ZnPc-RBS under different irradiation conditions, as shown in Figure 4D. Under 980 nm light, NO signal is observed to be steadily increased while little NO is produced under 1550 nm or 808 nm light excitation. Clearly, the results in Figures 4C and 4D show that the release of ROS and NO can only be activated by 1550 nm and 980 nm light, respectively. The independent control of ROS and NO generation is quite important as it offers a convenient tool for sequential photoactivation of NO and ROS release, which is also critical for programmed therapeutic applications.
Programmed therapeutic processes *in vitro*

Prior to use for therapeutic applications, it is necessary to test the cytotoxicity of UCNPs@mSiO2-ZnPc-RBS nanocomposites. HeLa cells were incubated with UCNPs@mSiO2-ZnPc-RBS at different concentrations (0, 50, 100, and 200 μg/mL) for 24 h, and the cell viability was evaluated using the CCK-8 assay. As shown in Figure 4E, the cell viability does not change significantly as the concentration of UCNPs@mSiO2-ZnPc-RBS increases, indicating good biocompatibility of as-developed UCNPs@mSiO2-ZnPc-RBS nanocomposites. Additionally, confocal laser scanning microscopy (CLSM) was used to image the intracellular uptake of UCNPs@mSiO2-ZnPc-RBS nanocomposites. Lyso-Tracker and Mito-Tracker dye was used to stain lysosomes and mitochondria, respectively, while green luminescence upon 808 nm light illumination was used to collect the signal of UCNPs@mSiO2-ZnPc-RBS nanocomposites. The upconversion luminescence of UCNPs@mSiO2-ZnPc-RBS gradually overlaps with lysosome (Figure S8A) and mitochondria (Figure S8B) signals as the incubation time increases. As shown in Figure S8C, the Pearson’s correlation coefficient for the UCNP and lysosomes is continuously increased from 0.10 after 2 h of incubation to 0.48 after 24 h of incubation. Similar increase of Pearson’s correlation coefficient can also be observed between UCNPs and mitochondria (Figure S8D). These results clearly show that the as-prepared UCNPs@mSiO2-ZnPc-RBS nanocomposites can be effectively taken up by HeLa cells.

Next, the oxygen consumption in the cell culture was measured in order to determine the role of NO in inhibiting cellular respiration activities. Experimentally, HeLa cells were first incubated with UCNPs@mSiO2-ZnPc-RBS for 24 h in the dark, which were then treated by 808 nm, 980 nm, and 1550 nm light (1 W/cm², 10 min), respectively. Figure 4F presents the oxygen consumption in the cell culture, which explicitly demonstrates that the culture medium illuminated by 980 nm light has the highest oxygen loss. It is believed that the NO released upon 980 nm light can inhibit the mitochondrial respiratory chain, thereby reducing oxygen consumption. It should be noted that the inhibitory control of NO on mitochondrial respiration is quite important as more oxygen can be saved to combat the hypoxia environment in the tumor region and accordingly promote the generation of ROS in the PDT modality.

In order to further verify the generation of NO intracellularly, HeLa cells were cultured with UCNPs@mSiO2-ZnPc-RBS nanocomposites, illuminated by the 980 nm light (1 W/cm²) for 10 min, and then imaged using CLSM at 0 min, 15 min, 30 min, 45 min, 60 min, and 90 min after the 980 nm light was turned off. From Figure 5A, it can be seen that the intracellular NO signal gradually increases over time. Particularly, the NO signal within 30 min is still relatively small while adequate amount of NO can be observed at 60 min after illumination. These results demonstrate that it takes time for the NO to be efficiently released and distributed inside cells, stressing the necessity of step-by-step activation of NO and ROS release. This is because if NO and ROS are released at the same time, NO may have limited time to bind to the mitochondria and the generation of ROS may still be hampered in the hypoxic environment. Figure 5B compares the intracellular ROS signals under different excitation conditions in hypoxia and normoxia environments. As expected, it is the highest using the sequence of 980 nm→1550 nm (980 nm light first and then 1550 nm light) under hypoxia condition. The quantitative analysis of the luminescence intensity further confirms that the ROS intensity produced under hypoxia environment via the sequence of 980 nm→1550 nm is about 2 times stronger than that of the 1550 nm→980 nm (1550 nm light first and then 980 nm), and about 2.7 times than the 1550 nm + 980 nm (1550 nm and 980 nm light at the same time).

Next, the influence of NO release on the cellular function is investigated. Specifically, a probe of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) was used to evaluate the mitochondrial membrane potential after NO treatment. In general, JC-1 exists in two forms: JC-1 monomer and JC-1 aggregate. In healthy cells, JC-1 enters the mitochondrial and forms JC-1 aggregates, emitting strong red color. While in unhealthy or apoptotic cells, JC-1 remains in the cytoplasm in its monomeric form, displaying green color. As shown in Figure S9A, for the NO-treated cells, strong green signals can be observed, in a comparison with the control group. This clearly indicates that the release of NO under normoxia by 980 nm light can lead to the depolarization mitochondrial membrane potential and cause mitochondrial damage. In light of the influence of NO and mitochondria function, we have then evaluated the generation of ATP (adenosine triphosphate) after NO treatment. Figure S9B indicates that, the NO treatment, via excitation by 980 nm light, results in much lower ATP content compared with groups treated with PBS and no light excitation. This is because the release of NO depolarizes mitochondrial membrane and reduces the membrane potential, which consequently affects cellular respiration and diminishes ATP production. Additionally, the intracellular oxygen consumption is closely related with the activity of an oxidase, called cytochrome c oxidase (CcO), which can convert oxygen into water through the respiration chain. Therefore, the CCO assay is then used to evaluate its activity. As shown in Figure S9C, the CCO activity is significantly suppressed in cells treated with NO (UCNPs@mSiO2-ZnPc-RBS+980 nm light) compared to the other two groups (PBS and UCNPs@mSiO2-ZnPc-RBS). This is because the released NO can bind to the CCO and affect its activity, thereby inhibiting cellular respiration.

The specially developed programmable activation of phototherapeutics was then evaluated based on the cell viability in vitro. HeLa cells were first incubated with UCNPs@mSiO2-ZnPc-RBS at different concentrations (0, 50, 100, and 200 μg/mL) and then treated by 808 nm light, 980 nm light, 1550 nm light, and their different combinations under normoxia (Figure 5C) and hypoxia conditions (Figure 5D).

As shown in Figures 5C and 5D, the cell viability of HeLa cells treated with 808 light excitation can still remain around 100%, indicating that the 808 nm light has negligible influence on the cell viability. Therefore, the green emission released upon 808 nm light illumination can be used as an imaging tool for diagnostic purpose. For the cells treated with 980 nm light, the generated nitric oxide (NO) can disturb cell metabolism and lead to oxidative stress destruction and free radicals. As a result, the reduction of cell viability can be observed in both normoxia and hypoxia environments. Upon illumination by 1550 nm light only, the cell viability is found to be below 40% under normoxia condition when HeLa cells were cultured with 200 μg/mL of UCNPs@mSiO2-ZnPc-RBS, indicating adequate amount of ROS is generated during the PDT process. However, the cell viability is increased to around 50% under hypoxia condition. Similar results are also observed for the cells treated with 1550 nm light first and then 980 nm (1550 nm→980 nm), and with 1550 nm and 980 nm light at the same time (980 nm + 1550 nm).
The increase of cell viability in the hypoxia environment can be attributed to the reduction of oxygen content and accordingly less generation of ROS. Conversely, for the HeLa cells treated using 980 nm light first and then 1550 nm light (980nm/1550 nm), the cell viability is only slightly increased from 23% under normoxia condition to 28% under hypoxia condition. Furthermore, the same trend is also consistent with the quantitative analysis of flow cytometry (Figure S10). Notably, the establishment of hypoxia condition is mainly used to mimic the oxygen-deficient environment in the tumor region. It is rather clear that the ROS generation in PDT is greatly affected by the oxygen presence in the surrounding environment. Under this circumstance, the programmable approach, 980 nm/1550 nm, has demonstrated outstanding performance in combating the hypoxia obstacle. Upon excitation by 980 nm light, NO is firstly released, which can then bind to the mitochondria and hence inhibit the cellular respiration. Therefore, more oxygen can be saved for the generation of ROS in PDT activated by the subsequent 1550 nm light.

**Programmed phototherapy in vivo**

For the programmed phototherapy in vivo, the female nude mouse was used as an animal model. First, the mice were subcutaneously injected with 4T1 tumor cells. Although the internalization of UCNPs within living organisms depends on their size and surface ligands, the UCNPs with the size range of 50~250 nm are internalized via endocytosis mechanism. Once inside the cells, UCNPs may rapidly undergo morphological transformation in the physiological environment, such as the acidic medium of lysosomes. Another previous study has also reported that UCNPs may be decomposed (dissolved) and excreted via feces and urine. As a result, the UCNP-based solution is injected into the mouse every three days during the treatment. When the tumor grew to about 100 mm³ in size, the mice were randomly divided into eight groups with five mice in each group: control group 1 (PBS solution), control group 2 (UCNPs@mSiO₂-ZnPc-RBS), group 3 (808 nm), group 4 (980 nm), group 5 (1550 nm), group 6 (1550 nm → 980 nm), group 7 (1550 nm + 980 nm), and group 8 (980 nm → 1550 nm). The laser power density is 1 W/cm², and the illumination time for each group is 20 min in total. During the 14 days of treatment, the body weight and tumor size were measured every 3 days.
other day. Figure 6A compares the growth of tumors in different groups after treatment. It can be seen that the tumors in the control group, group 1, group 2 and group 3 grow rapidly, while the growth of tumors in groups 4, 5, 6, and 7 is slightly inhibited. In contrast, the tumor size in group 8 is the smallest, indicating the tumor growth is greatly inhibited by the programmed phototherapy under the sequence of scheduling NO release first, and then ROS.

Figure 6B shows the changes of relative body weight in eight groups within 14 days. NO significant decrease or increase in the body weight is observed, indicating that the as-developed nanocomposites have good biocompatibility. After 14 days of treatment, the mice in all groups were sacrificed and the tumor tissues were stripped. As shown in Figure 6C, the tumor volume is the smallest for the mice group treated with the light illumination in the sequence of 980 nm / 1550 nm. In order to further prove the therapeutic effect, we also performed histological hematoxylin-eosin staining (H&E) staining analysis on different groups of tumors and major organs. Figure 6D presents the H&E stained images of tumor tissue sections. Compared with other groups, most severe apoptosis or necrosis and nucleus contraction are also observed in the group of 980 nm / 1550 nm Figure S11 shows the H&E stained images of main organs (heart, liver, spleen, lung, and kidney) of mice in different groups. No evident damages are observed, which indicates that the as-prepared UCNPs@mSiO₂-ZnPc-RBS has no short-term toxicity and demonstrates great potential for clinical applications.

DISCUSSION

Due to their deeper tissue penetration depth and unique optical properties, lanthanide-doped UCNPs have been widely used as light transducers to excite photosensitizers for many types of phototherapeutic applications, such as PDT, gas therapy, chemotherapy, and their combinational therapies. Taking PDT as an example, UCNPs have been widely employed to activate PDT photosensitizers for release of ROS since the first reported study. As for the gas therapy, application of UCNPs to trigger NO donors to generate NO was initially demonstrated by Zhang et al. in 2015. A summary of the progress of NO-involved therapy based on UCNP nanocomposites is presented in Table S1. It indicates that NO has been used to alleviate tumor hypoxia or overcome multidrug resistance in combination with PDT or chemotherapy. Moreover, NO can trigger the immune response for NO-activated immunotherapy or enhance radiotherapy as an effective hypoxic radiosensitizer. However, in previous studies, NO and other therapeutic molecules (e.g., ROS and DOX (doxorubicin)), are mainly released simultaneously rather than in a programmable manner during the photoactivation process. For an ideal phototherapy treatment, it should be methodically optimized and each of its key processes should be controlled in a sequential manner, which means that appropriate photoactivation should be administered at the appropriate time and for the appropriate duration. However, such level of accuracy in UCNP-based
phototherapy is limited because previously reported UCNPs do not allow for separate photoactivation of different therapeutic processes, as they are commonly responsive to only one excitation wavelength.

One approach to address this issue is to develop orthogonal emissive UCNPs that can emit different colors in response to different excitation lights. For example, these orthogonal emissive UCNPs have been used for cell imaging and phototherapy independently. However, in these studies, the UCNPs can only respond to two NIR excitation lights and programmable control over more complicated processes is still lacking. The active-core/active shell approach has been successfully applied to tune the upconversion luminescence in lanthanide-doped nanoparticles. In this study, we have employed an energy segmentation-based strategy to construct core-multi-shell structured UCNPs that enable to release orthogonal trichromatic colors in response to three NIR lights. Because more excitation and emission profiles are independently controlled, the as-developed UCNP-based nanoplatform allows for spatiotemporal control over more than two processes in a PDT treatment, including imaging diagnostics, suppression of tumor hypoxia and release of ROS. As a result, the treatment efficiency is significantly enhanced via such a stepwise manner. It is believed that the as-reported nanoplatform represents a high-level precision of phototherapy and would be useful for programming and customized cancer treatment.

Conclusions
In this study, orthogonal emissive UCNPs, NaYF₄@NaErF₄:Tm@NaYF₄@NaYF₄:Yb,Ho,Nd@NaYF₄@NaYF₄:Yb,Tm, are reported. By precisely tuning the thickness of luminescent and inert shells, the as-developed UCNPs can release orthogonal trichromatic colors in response to three NIR lights, i.e., red emission upon 1550 nm light excitation, green emission upon 808 nm light excitation, and blue emission upon 980 nm light excitation. As a proof of concept, these photoswitchable UCNPs are further incorporated with the photosensitizer (Zinc phthalocyanine, ZnPc) and NO donor (RBS to construct an intelligent “off-on” theranostic PDT nanoagent. Thanks to the orthogonal emissive properties of UCNPs, imaging guidance, NO generation, and ROS release can be triggered independently upon alternating irradiation with 808 nm light, 980 nm light, and 1550 nm light, allowing for a cascaded therapeutic modality. The results demonstrate that the sequential activation of NO and PDT can dramatically alleviate tumor hypoxia and greatly enhance the PDT efficiency, which is believed to have a new avenue for future clinical phototherapeutic treatment.

Limitations of the study
This study uses an energy segmentation-based strategy to synthesize trichromatic UCNPs. A notable limitation lies in the requirement for five shells to be applied onto the core nanoparticle to achieve distinct and orthogonal emissions using different excitation lights. Clearly, this makes the fabrication rather challenging and time-consuming. Future research could explore alternative and more convenient strategies to simplify the nanoarchitecture by using fewer shells.

Besides, the phototherapeutic process during the PDT treatment does not only involve the consumption of oxygen and generation of ROS. As a result, more detailed investigation is currently underway to study the PDT treatment on the biochemical level, thereby enhancing the understanding of its underlying mechanisms.

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

S.Z., H.Z., and T.S. contributed equally to this work. X.Z. and Y.Z. designed this study; S.Z., H.Z., T.S., Y.X., J.W., Y.T., Y.W., and J.L. performed the research; All authors analyzed and interpreted the data; S.Z., H.Z., T.S., and X.Z. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES


STAR METHODS

KEY RESOURCES TABLE

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Xiaohui Zhu (xhzhu@shu.edu.cn).

Materials availability
No new materials were created during this study.

Data and code availability
- Data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals and tumor models
Female nude mice (4–5 weeks old) were provided by the Shanghai Laboratory Animal Center (SLAC, Shanghai). In order to build a in vivo tumor model, 4T1 tumor cells were subcutaneously injected into the mice. When the tumors grew to about 100 mm³ in size, the in vivo treatment was conducted. All animal-related experiments were performed with the guidelines of the Institutional Animal Care and Use Committee of Shanghai University.
Cell culture
Human cervical cancer cells (HeLa cell) were cultured in DMEM medium containing 10% FBS (fetal bovine serum), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C incubator. HeLa cells were seeded in small dishes at a density of 1.5×10⁵ cells per dish for 24 h and incubated with samples for 24 h subsequently.

METHOD DETAILS
Preparation of core-multi-shell UCNPs

Step 1: Synthesis of the core nanoparticles
1.0 mmol YCl₃ aqueous solution was added to a 100 mL three-necked flask, and the temperature was raised to 120°C. Then, 6 mL oleic acid (OA) and 15 mL 1-octadecene (ODE) were added and heated to 150°C with constant stirring for 30 min to form the lanthanide–oleic acid complex, which was then cooled down to 60°C. 8 mL methanol solution containing 100 mg NaOH and 75 mg NH₄F was added to the flask and heated to 120°C for 35 min. Thereafter, the temperature was rapidly increased to 300°C under Ar atmosphere for 1 h. After the reaction was complete, the nanoparticles were washed with cyclohexane/ethanol several times. Finally, the products were stored in 20 mL cyclohexane.

Step 2: Synthesis of core-shell UCNPs
0.199 mmol ErCl₃ and 0.001 mmol TmCl₃ were added to 100 mL three flasks. Then, the temperature was raised to 120°C. 6 mL oleic acid (OA) and 15 mL 1-octadecene (ODE) were added and heated to 150°C for 30 min. Next, cyclohexane solution containing 40 mg core nanoparticles was injected into the flask, kept at 150°C to remove cyclohexane and then cooled down to 60°C. 8 mL methanol solution containing 50 mg NaOH and 75 mg NH₄F were added to the flask and heated to 120°C for 35 min. Thereafter, the temperature was rapidly increased to 300°C under Ar atmosphere for 1 h to grow core-shell nanoparticles. After the reaction was complete, the nanoparticles were washed with cyclohexane/ethanol several times and redispersed in cyclohexane.

Step 3: Synthesis of core-shell-shell UCNPs
The synthesis of NaYF₄@NaErF₄:Tm@NaYF₄(Core@S1@S2) was similar to that of Core@S1 nanoparticles, except that the Core@S1 nanoparticles were used as seeds while YCl₃ was used as the shell precursor. Besides, the thickness of S2 shell could be varied by controlling the amount of YCl₃ precursor.

Step 4: Synthesis of core-shell-shell-shell UCNPs
The synthesis of NaYF₄@NaErF₄:Tm@NaYF₄@NaYF₄@Yb,Ho,Nd (Core@S1@S2@S3) synthesis was similar to that of Core@S1@S2 nanoparticles, except the Core@S1@S2 nanoparticles were used as seeds while YCl₃, YbCl₃, HoCl₃, and NdCl₃ were used as shell precursor.

Step 5: Synthesis of core-shell-shell-shell-shell UCNPs
The synthesis of NaYF₄@NaErF₄:Tm@NaYF₄@NaYF₄@NaYF₄@Yb,Ho,Nd (Core@S1@S2@S3@S4) was similar to that of Core@S1@S2@S3 nanoparticles, except that the Core@S1@S2@S3 nanoparticles were served as seeds while YCl₃ was used as the S4 precursor.

Step 6: Synthesis of core-shell-shell-shell-shell-shell UCNPs
The synthesis of NaYF₄@NaErF₄:Tm@NaYF₄@NaYF₄@NaYF₄@NaYF₄:Yb,Tm (Core@S1@S2@S3@S4@S5) was similar to that of Core@S1@S2@S3@S4 nanoparticles, except that the Core@S1@S2@S3@S4 nanoparticles were served as seeds while YCl₃, YbCl₃, and TmCl₃ was used as the S5 precursor.

Preparation of mesoporous silicon modified UCNPs (UCNPs@mSiO₂)
Firstly, 3 mL as-prepared UCNPs (approximately 45 mg) was added to 20 mL ultra-pure water containing 0.3 g CTAB. The solution was then vigorously stirred overnight to evaporate the cyclohexane. Next, 40 mL distilled water, 6 mL ethanol, 300 μL NaOH (2 M) was added successively to a 250 mL three-neck flask and heated to 70°C stirring for 30 min. Thereafter, 400 μL TEOS was slowly added drop by drop under vigorous magnetic stirring. The reaction was cooled down to room temperature. The products were centrifuged and washed 3-4 times with ethanol. In order to remove the CTAB template, the precipitate was dissolved in 40 mL acidic ethanol solution (pH~1.5) and stirred at 70°C for 3 h. The above etching process was repeated three times and the obtained precipitate, UCNPs@mSiO₂, were finally stored in 10 mL ethanol.

Loading ZnPc and RBS photosensitizers into UCNPs@mSiO₂
5 mg UCNPs@mSiO₂, 1.2 mg ZnPc and 1.2 mg RBS were dissolved in 2 mL pyridine solution and stirred for 12 h under dark conditions. The precipitate was centrifuged and washed three times with deionized water. The obtained product (UCNPs@mSiO₂-ZnPc-RBS) is dispersed in deionized water.
Detection of ROS

1,3-diphenylisobenzofuran (DPBF) probe was used to detect the ability of UCNPs@mSiO2-ZnPc to release ROS. Under dark conditions, 10 μL ethanol solution of DPBF (10 mM) was mixed with 2 mL UCNPs@mSiO2-ZnPc-RBS solution. The solution was then irradiated with 808 nm, 980 nm and 1550 nm lasers for 30 min, respectively. The UV-Vis absorption of DPBF was tested every 5 min. Meanwhile, control experiments were also performed in the pure DPBF and laser-free irradiation groups.

The generation of intracellular ROS was performed by using DCFH-DA as a probe. Human cervical cancer cells (HeLa cell) were cultured in DMEM medium containing 10% FBS (fetal bovine serum), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C incubator. HeLa cells were seeded in small dishes at a density of 1.5 x 10^5 cells per dish for 24 h and incubated with UCNPs@mSiO2-ZnPc-RBS (2 mL) for 24 h subsequently. The DCFH-DA probe was added to small dishes and placed into an incubator for 30 min, and cells were washed multiple times with PBS. The small dishes were then placed in or not in a sealed box with anaerobic gas producing bags (5% CO2 and less than 1% O2) and incubated for 1 h to mimic normoxic or hypoxia conditions in the dark. Cells in the corresponding small dishes were irradiated by 808 nm light (1 W/cm², 10 min), 980 nm (1 W/cm², 10 min), 1550 nm (1 W/cm², 10 min), and their combinations with different sequences (980 nm + 1550 nm, 980 nm → 1550 nm, and 1550 nm → 980 nm). Notably, 980 nm + 1550 nm means that cells were irradiated by 980 nm and 1550 nm for 10 min simultaneously, 980 nm → 1550 nm means that cells were firstly irradiated with 980 nm for 5 min, followed by 1550 nm for another 5 min, and 1550 nm → 980 nm means that cells were firstly irradiated with 1550 nm for 5 min, followed by 980 nm for another 5 min.

Detection of nitric oxide

A commercially available Griess detection kit was used to detect NO. UCNPs@mSiO2-ZnPc-RBS containing solutions were irradiated with 808 nm, 980 nm and 1550 nm light for 30 min, respectively. Then, 200 μL of suspension was extracted every 5 min, and the supernatant was retained by centrifugation. Griess reagent I and Griess reagent II were then added. Whereafter, the TecanSpark multifunctional microplate reader was used to measure the fluorescence intensity of the mixed solution at a wavelength of 540 nm. Meanwhile, the non-laser irradiation group was used as a control experiment. Intracellular NO was detected using a commercial DAF-FMDA fluorescence probe. HeLa cells were first incubated with UCNPs@mSiO2-ZnPc-RBS for 24 h, stained with DAF-FMDA for 30 min. Then, the cells were treated by 980 nm laser irradiation (1 W/cm², 10 min) and then observed under a CLAM at 0,15,30,45,60, and 90 min after irradiating.

Measurement of oxygen content in cell culture

HeLa cells were seeded in small dishes at a density of 1.5 x 10^5 cells per dish for 24 h and incubated with UCNPs@mSiO2-ZnPc-RBS (2 mL) for 24 h. Small dishes were placed in an anaerobic nitrogen glove box and then irradiated with 808 nm, 980 nm and 1550 nm (1 W/cm², 10 min) laser, respectively. After that, the oxygen content was recorded every 5 min using oxygen electrodes. The relative oxygen content was calculated as the ratio of the oxygen content measured at each time point to that measured at the initial stage.

Confocal imaging of cellular uptake of UCNPs@mSiO2-ZnPc-RBS

HeLa cells were seeded in small dishes at a density of 1.5 x 10^5 cells per dish for 24 h and incubated with UCNPs@mSiO2-ZnPc-RBS (2 mL) for 2 h, 8 h, 12 h, and 24 h, respectively. Lyso-Tracker-Green and Mito-Tracker-Red dye were added to the cells to stain the lysosome and mitochondria. Then, cells were washed multiple times with PBS and cellular uptake imaging was performed using a confocal laser scanning microscope.

Evaluation of apoptosis

HeLa cells were seeded in a 6-well culture plate at a density of 1.5 x 10^5 cells per well for 24 h, and incubated with UCNPs@mSiO2-ZnPc-RBS for 24 h. The 6-well plate was then placed in or not in a sealed box containing anaerobic gas producing bags (5% CO2 and less than 1% O2) to simulate hypoxia or normoxic conditions in the dark. Cells in the corresponding small dishes were respectively irradiated with 808 nm, 980 nm, 1550 nm, 980 nm → 1550 nm, 1550 nm → 980 nm and 980 nm + 1550 nm (1 W/cm², 10 min) laser light, and then incubated in the incubator for another 4 h. After that, cells were transferred into a centrifuge tube, centrifuged at 900 r/min for 5 min and washed twice by PBS. The cells were added into a 500 μL of buffer, stained using Annexin V-FITC and PI for 20 min under dark conditions, and then measured by flow cytometry.

Assessment of cytotoxicity and in vitro PDT treatment

HeLa cells were seeded in a 96-well culture plate at a density of 8000 cells per well for 24 h. A series of culture medium solutions with 100 μL UCNPs@mSiO2-ZnPc-RBS (0, 50, 100, and 200 μg/mL) were added to cell well plates for 24 h. Then, the CCK-8 assay was used to evaluate the cell viability. For the in vitro PDT experiment, the cells were incubated with different concentrations of UCNPs@mSiO2-ZnPc-RBS for 24 h in 96-well plates. The 96-well plate was then placed in or not in a sealed box containing anaerobic gas producing bags (5% CO2 and less than 1% O2) to simulate hypoxia or normoxic condition. Cells in the corresponding 96-well plates were respectively irradiated with 808 nm, 980 nm, 1550 nm, 980 nm → 1550 nm, 1550 nm → 980 nm and 980 nm + 1550 nm (1 W/cm², 10 min) laser light, and then incubated in the incubator for another 24 h. 10 μL CCK-8 reagent was subsequently added to each well plate and incubated in the incubator for another 2 h. The absorbance at 450 nm was recorded in a Tecan Spark multifunctional microplate reader.
**In vivo PDT/NO therapy experiments in mice**

First, mice were injected with 4T1 tumors subcutaneously. When the tumors grew to about 100 mm³ in size, the mice were randomly divided into eight groups with five mice in each group: control group 1 (PBS solution), control group 2 (UCNPs@mSiO₂-ZnPc-RBS), group 3 (UCNPs@mSiO₂-ZnPc-RBS with 808 nm), group 4 (UCNPs@mSiO₂-ZnPc-RBS with 980 nm), group 5 (UCNPs@mSiO₂-ZnPc-RBS with 1550 nm), and group 6 (UCNPs@mSiO₂-ZnPc-RBS with 1550 nm + 980 nm), group 7 (UCNPs@mSiO₂-ZnPc-RBS with 1550 nm → 980 nm), and group 8 (UCNPs@mSiO₂-ZnPc-RBS with 980 nm → 1550 nm). The laser power density is 1 W/cm², and the illumination time for each group is 20 min in total. The body weight and tumor size were measured every other day within the 14 days of treatment. Relative body weight was calculated as m/m₀, where m₀ and m indicates body weight before and after treatment, respectively. Tumor volume was calculated as follows: Tumor volume (v) = (length × width²)/2. Relative tumor volume was calculated as v/v₀ (where v₀ is the tumor volume at the beginning of treatment). After 14 days of treatment, the main organs (heart, liver, spleen, lung, and kidney) and tumor tissues of the eight groups of mice were H&E stained and imaged by fluorescence microscopy.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Standard error of the mean is shown on figures unless otherwise noted. The study does not include any specific quantification and statistical analysis.