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Irradiation-free photodynamic therapy *in vivo* induced by enhanced deep red afterglow within NIR-I bio-window

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HIGHLIGHTS

- Realizing an irradiation-free PDT using deep red intense afterglow nanoparticles.
- This PDT merely needs one-shot irradiation before PDT treatment.
- The required $^1\text{O}_2$ in PDT process can be induced by enhanced deep red afterglow.
- The inhibition factor of cancer growth is as high as ~80% after this PDT treatment.
- PDT effect can be consolidated by red light reirradiation with extremely low power.

GRAPHICAL ABSTRACT

Through nanopatform constructed using deep red persistent luminescent nanoparticles (DRPLNPs), an irradiation-free PDT is demonstrated *in vitro* and *in vivo*. Strikingly, $^1\text{O}_2$ molecules induced by enhanced deep red afterglow emissions play a key role in inhibiting the tumor growth. The inhibition factor of cancer growth two weeks after the injection of the pre-irradiated (635 nm light) nanopatforms is as high as ~80%.

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ABSTRACT

In situ precise anti-cancer therapeutics, such as imaging-guided photodynamic therapy (PDT), is one of the major efforts in reducing the mortality caused by tumor proliferation. Up to now, PDT can only be executed in hospital because of the restriction of relevant facilities and PDT specialists, e.g. lasers whose power density needs to be adjusted to a patient tolerable level on time. Therefore, an irradiation-free and long-term PDT is highly desirable in precision medicine, which may help for the realization of PDT at home for cancer patients. Herein, based on the facilitated tunneling process induced by Bi substitution in Cr doped zinc gallogermanate (ZGGO:Cr,Bi) deep red persistent luminescent nanoparticles (DRPLNPs) within NIR-I bio-window (650–950 nm), an irradiation-free PDT nanopatform was constructed by loading zinc phthalocyanine (ZnPc) molecules into mesoporous silica coated on the surface of ZGGO:Cr,Bi DRPLNPs (ZGGO:Cr,Bi@mSiO₂-ZnPc). It was demonstrated *in vitro* and *in vivo* that Bi substitution can effectively improve the afterglow intensity to such that the production of $^1\text{O}_2$ is sufficient to have an apparent irradiation-free PDT. The inhibition factor of cancer growth two weeks after the

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injection of the nanocomposites was as high as ~80%. This work exhibits the interesting potential of the new generation of afterglow nanomaterials in precision medicine.

1. Introduction

To reduce the high mortality caused by tumor proliferation, precision cancer diagnostics and therapy becomes one of the hotspot issues [1]. In particular, imaging-guided photodynamic therapy (PDT) is attracting more attentions due to its potential applications in cancer therapy [2–7]. Usually, the corresponding therapeutic nanoplatforms consist of luminescent/fluorescent nanomaterials for bioimaging and nontoxic photosensitizers served as therapeutic agents [5,8–12]. In case of optical imaging-guided PDT, the external X-ray, visible or near infrared (NIR) light radiation is required to excite the luminescent nanomaterials, such as quantum dots and lanthanide-doped materials, for optical imaging [4,6,8,13–15]. The anchored photosensitizers (PS), e.g. zinc phthalocyanine (ZnPc) and Chlorin e6 (Ce6), generate reactive oxygen species (ROS) to execute PDT in which the nanomaterials are played as light transducers [6,16–18]. Although PDT assisted by these nanophotosensitizers exhibits unique advantages compared to traditional sensitizers, such as minimum invasive treatment, low side effects, no cumulative radiation dose and real-time visible diagnosis [19–21], the demand of external excitation light source makes it difficult for imaging and treating cancers in deep tissue. Most lethally, during the intermission of cancer therapy, i.e. after ceasing X-ray, visible or deep red light irradiation, it is still a critical issue on how to avoid the rebound tumor growth without the secondary therapy and how as far as possible to abandon external radiation for improving the life quality of cancer patients physically and mentally [22–25].

With the advent of nanotechnology, it is nowadays possible to acquire autofluorescence-free biosensing/bioimaging for theranostics using engineering related to persistent luminescent nanoparticles, which emission wavelength falls in the so called NIR first biological window (650–950 nm, NIR-I bio-window [26–29]) after stopping external light excitation [30–37]. For example, a renewable deep red bioimaging was realized using $\text{ZnGa}_2\text{O}_4\text{:Cr}$ (ZGO:Cr) DRPLNPs after 254 nm irradiation (6 W) followed by a 650 nm re-excitation (~5000 lm) because of the absorption band edge related to the $^4\text{A}_2 \rightarrow ^4\text{T}_2$ transition of Cr^{3+} , i.e. part of absorption wavelengths, falling in the 600–650 nm region [35,37–39]. More recently, metastasis tracking and photodynamics-assisted chemotherapy were acquired by constructing Cr doped zinc gallogermanate (ZGGO:Cr^{3+}) nanoplatform, i.e. $\text{ZGGO:Cr,Yb,Er@hSiO}_2\text{@CCM}$, under UV light irradiation (254 nm, 6 W) in combination with a following 650 nm (~2000 lm) and 808 nm light re-excitation (3 W cm^{-2}), respectively [40]. However, the afterglow intensity is till now not sufficient to induce photodynamic therapy directly, as alternatives all the reported cases also require the regular recovery of afterglow intensity via X-ray or red light irradiation with higher power (such as $\sim 1 \text{ W cm}^{-2}$) during a PDT program [41–43]. These approaches in fact reduce significantly the advantage of employing persistent light, i.e. long duration of PDT without external stimuli.

To circumvent the drawback of these approaches the key issue is to enhance the intensity of afterglow to such that ROS is produced sufficiently. It was found that the doped cations of which the valence states may readily change, such as bismuth (Bi) ions, can serve as electron traps and enhance the persistent luminescence [44,45], thus Bi element might be an appreciate doping agent to enhance the afterglow emission intensity of ZGGO:Cr^{3+} nanoparticles.

In this paper, we report the realization of PDT with the aid of an improved afterglow nanoplatform. Bi and Cr codoped zinc gallogermanate (ZGGO:Cr,Bi) nanoparticles with the deep red afterglow emissions at ~700 nm and the afterglow time of over 900 min were

prepared by a modified sol-gel method in combination with a subsequent vacuum annealing. Bi substitution facilitates the tunneling processes from deep traps to the $^4\text{T}_2$ level of Cr^{3+} and leads to an enhanced deep red afterglow emission, even under red light (~635 nm) excitation. *In situ* deep red afterglow nanosensitizers were constructed by loading ZnPc molecules into mesoporous silica coated the surface of ZGGO:Cr,Bi DRPLNPs ($\text{ZGGO:Cr,Bi@mSiO}_2\text{-ZnPc}$). Red light excitation has two channels in executing PDT, one is the direct excitation of photosensitizers loaded on the surface of nanoparticles, and the other is, after stopping the irradiation of red light, the photosensitizers is excited by the afterglow of the nanoparticles. The efficacy of the latter was tested *in vivo*, and the inhibition factor as high as 80% was obtained in two weeks after the intratumor injection of the solution containing pre-irradiated $\text{ZGGO:Cr,Bi@mSiO}_2\text{-ZnPc}$. The realization of PDT induced by deep red afterglow may pave the way towards an irradiation-free PDT of cancer.

2. Materials and methods

2.1. Synthesis of ZGGO:Cr,Bi DRPLNPs

ZGGO:Cr,Bi DRPLNPs were prepared by a modified sol-gel method and following a vacuum-annealing. The raw materials, $\text{Zn}(\text{CH}_3\text{COO})_2$, $\text{Ga}(\text{NO}_3)_3 \cdot x\text{H}_2\text{O}$, GeCl_4 , $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ and $\text{Bi}(\text{CH}_3\text{COO})_3$ (Sigma-Aldrich) were mixed together according to mole ratio of $\text{Zn:Ga:Ge:O:Cr:Bi} = 2:2.98:0.75:8:0.02:x$ ($x = 0, 0.02, 0.04, 0.08, 0.12, 0.16$). After the mixture heated at 453 K for 4 h in the steel autoclave, the obtained wet gel precipitates were washed three times with deionized water and dried at 353 K for 10 h. In vacuum, the xerogels with a certain Bi doping concentration were annealed at 750 and 800 °C for 1.5 h, respectively. The final $\text{Zn}_2\text{Ga}_{2.98}\text{Ge}_{0.75}\text{O}_8\text{:Cr}_{0.02}^{3+}\text{Bi}_x^{3+}$ (ZGGO:Cr,Bi) nanoparticles were acquired after grinding the annealed xerogels softly.

2.2. Characterization

Crystal structure of ZGGO:Cr,Bi DRPLNPs was examined by an Rigaku XRD spectrometer (D/max-RA) using $\text{CuK}\alpha$ radiation (line of 0.15418 nm). The change in the morphology of ZGGO:Cr,Bi DRPLNPs as a function of Bi doping concentration was investigated by a field emission transmission electron microscope (TEM, JEOL-2100F). The energy dispersive X-ray (EDX) spectrum was carried out using a scanning electron microscope (SEM, FEI, Quanta FEG 250). The photoluminescence spectra were recorded by a SHIMADZU spectrophotometer (RF-5301PC) with a R928 PMT detector. The luminescence kinetic measurement was performed by using an optical parametric oscillator (OPO) system with a digital oscilloscope (600 MHz, LeCroy). The afterglow decay curves were carried out using a spectrophotometer (Omin λ -300, Zolix) after 635 nm laser irradiation. The ZGGO:Cr,Bi DRPLNPs were pressed into pellets to measure thermoluminescence spectra ($\lambda_{\text{mon}} = 696 \text{ nm}$, temperature range: 90–520 K) using an Instec heating and cooling stage (HCP621G). The heating rate was 10 K min^{-1} . Absorption spectra were performed using a UV-vis-NIR spectrophotometer (SHIMADZU, UV4150). Fourier transform infrared (FTIR) transmittance spectra were carried out using a FT-IR spectrometer (Thermo Electron, Nicolet 6700).

2.3. Synthesis of ZGGO:Cr,Bi@mSiO_2 nanoplatforms

The mesoporous silica coated ZGGO:Cr,Bi (ZGGO:Cr,Bi@mSiO_2)

nanoplatfoms were constructed by a two-step Stöber method. Firstly, 50 mg ZGGO:Cr_{0.02},Bi_{0.12} nanoparticles annealed at 750 °C were dispersed in ethanol (80 mL) and this solution was stirred for 1 h. Then 20 mL deionized water and 30 µL tetraethylortosilicate (TEOS, aladdin) were added into the mixed solution and stirred for 0.5 h. Then 600 µL ammonia solution (28 wt%) was added into the final mixed solution. After the final mixed solution continuously reacting for 12 h with a stir, an amorphous SiO₂ layer was coated to the surface of the ZGGO:Cr,Bi DRPLNPs. To remove the residual TEOS, the amorphous SiO₂ coated ZGGO:Cr,Bi (ZGGO:Cr,Bi@mSiO₂) nanoplatfoms were washed with the mix solution of water and ethanol. Secondly, the ZGGO:Cr,Bi@mSiO₂ nanoplatfoms were dispersed into the water/ethanol mixing solution (140 mL) with a volume (V) ratio of V_{water}:V_{ethanol} = 4:3. Then, 1 mL ammonia solution (28 wt%) and 0.3 g cetyltrimethylammonium bromide (CTAB, aladdin) were added into the ZGGO:Cr,Bi@mSiO₂ solution. Subsequently, 0.3 g TEOS was added into the reaction solution. After stirring and reacting for 12 h, the final resultants were washed with the water/ethanol mixing solution. Then the final dried resultants were dispersed into the mixing solution of HCl (37 wt%, 1 mL) and anhydrous ethanol (50 mL). The mesoporous ZGGO:Cr,Bi@mSiO₂ nanoparticles were acquired after a reflux process at 60 °C.

2.4. In vitro cytotoxicity assays

Mouse hepatoma (Hepa) 1–6 cells were cultured in a basal culturing medium containing Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) and supplementing 10% (vol/vol) fetal bovine serum first. Then the cultured Hepa 1–6 cells were plated in 96-well plates (8000–10000 cells/well) and incubated for 24 h in a humidified 37 °C environment (containing 5% CO₂). After Hepa 1–6 cells attaching to wall, they were exposed to different concentrations of ZGGO:Cr,Bi and ZGGO:Cr,Bi@mSiO₂ solutions (0, 12.5, 25, 50 and 100 µg mL⁻¹) for 24 h, respectively. Then, 10 µL MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) solution (5 mg mL⁻¹) was added to each well and the plates were incubated for 4 h in the humidified 37 °C environment. Finally, each well adds 100 µL DMSO (dimethyl sulfoxide, aladdin). The values of optical density (λ_{mon} = 490 nm) were measured using a microplate reader and the cell viability was calculated by the following equation: cell viability (%) = (absorbance value of treatment group/mean absorbance value of control) × 100%. All tests were performed in three independent experiments.

2.5. Loading photosensitizer into ZGGO:Cr,Bi@mSiO₂

Zinc phthalocyanine (ZnPc, Sigma-Aldrich) was selected as a photosensitizer and was loaded into the pores of the mesoporous silica layer after soaking ZGGO:Cr,Bi@mSiO₂ nanoplatfoms (100 mg) in a 50 mL solution of ZnPc in DMSO (0.2 mg mL⁻¹) for 48 h at 30 °C with continuous stirring. The ZnPc-loaded ZGGO:Cr,Bi@mSiO₂ nanoplatfoms (ZGGO:Cr,Bi@mSiO₂-ZnPc) were obtained by centrifugation. The amount of ZnPc loaded into the pores of ZGGO:Cr,Bi@mSiO₂ nanoplatfoms was determined using the method as reported by Idris et al. [8]. The absorption spectra of the aforementioned ZnPc solution without ZGGO:Cr,Bi@mSiO₂ nanoplatfoms were measured before and after loading procedure. The mass of ZnPc incorporated into the nanoplatfoms can be obtained based on the standard curve derived from the absorption spectra of the standard ZnPc solutions (DMSO solvent) and the absorbance difference between the 672 nm absorption peaks of ZnPc solutions before and after loading. The ZnPc loading efficiency (LE) can be calculated using the following Eq. (1):

$$LE\text{ wt}(\%) = (M_1/M_2) \times 100\% \quad (1)$$

where M₁ and M₂ refer to the mass of ZnPc incorporated into the nanoplatfoms and the mass of the nanoplatfoms, respectively.

2.6. Measurement of ¹O₂ quantum yield of ZGGO:Cr,Bi@mSiO₂-ZnPc

The ¹O₂ quantum yield of ZGGO:Cr,Bi@mSiO₂-ZnPc was determined by a chemical trapping method [46]. 1,3-Diphenylisobenzofuran (DPBF, Sigma-Aldrich) and methylene blue (MB, Sigma-Aldrich) were used as the ¹O₂ trapping agent and the standard photosensitizer, respectively. To avoid the inner-filter effect, the absorbances (at 635 nm) of ZGGO:Cr,Bi@mSiO₂-ZnPc and MB in ethyl alcohol (EtOH) were adjusted to ~0.065 OD. To measure absorption spectrum, 15 µL DPBF solution (2 mg mL⁻¹) was added into the MB/EtOH and ZGGO:Cr,Bi@mSiO₂-ZnPc/EtOH solution in dark place, respectively. Then absorption spectra of DPBF in ZGGO:Cr,Bi@mSiO₂-ZnPc/EtOH and MB/EtOH solutions as a function of irradiation time were acquired in the case of 635 nm laser excitation (irradiated power: 50 mW cm⁻²). By fitting the obtained irradiation-time dependent absorbance (λ_{max} = 411 nm) of DPBF dispersed in the measured ZGGO:Cr,Bi@mSiO₂-ZnPc/EtOH and MB/EtOH solutions, their decay times (τ_{ZnPc} and τ_{MB}) were obtained. Thus the ¹O₂ quantum yield of ZGGO:Cr,Bi@mSiO₂-ZnPc nanoplatfoms (Φ_{ZnPc}) can be calculated by the following Eq. (2):

$$\Phi_{ZnPc} = \Phi_{MB} \cdot (\tau_{MB}/\tau_{ZnPc}) \quad (2)$$

where the ¹O₂ quantum yield of MB (Φ_{MB}) is used as a reference value (0.49).

2.7. Intracellular ROS detection

ROS generation inside cells was detected using 20',70'-dichlorofluorescein-diacetate (DCFH-DA) ROS assay kit (Beyotime). Hepa1-6 cells were incubated for 24 h and then they were further exposed to ZGGO:Cr,Bi@mSiO₂-ZnPc solution (100 µg mL⁻¹) for 24 h in a humidified 5% CO₂ atmosphere at 37 °C. The aforementioned cells treated with ZGGO:Cr,Bi@mSiO₂-ZnPc solution were irradiated for 10 min using 635 nm laser (150 mW cm⁻²). Then DCFH-DA was loaded into these Hepa1-6 cells with a 30-min incubation. The loaded cells were washed twice with PBS and used for acquiring fluorescence images related to intracellular ROS induced by deep red afterglow of ZGGO:Cr,Bi@mSiO₂-ZnPc nanoplatfom.

2.8. Photodynamic therapy for cancer cells

After pre-incubating Hepa1-6 cells for 24 h, they were further exposed to different concentrations of the ZGGO:Cr,Bi@mSiO₂-ZnPc solutions (0, 12.5, 25, 50 and 100 µg mL⁻¹) for 24 h in a humidified 5% CO₂ atmosphere at 37 °C. Then the incubated Hepa1-6 cells were irradiated by a 635 nm laser (irradiation time: 10 min, irradiation power: 150 mW cm⁻²). After stopping 10-min irradiation, the cells were incubated at 37 °C for 4 h. Finally the related MTT assay was performed. The PDT effect of ZGGO:Cr,Bi@mSiO₂-ZnPc on Hepa1-6 cells was evaluated by the cell viability of the Hepa1-6 cells. In addition, the Hepa1-6 cells subjected to the aforementioned PDT assay was labeled with Live/Dead assay reagents as reported by Choi et al. [47]. The fluorescence images of labeled cells were required by using a confocal laser scanning microscope (Olympus FV-1000).

2.9. In vivo deep red afterglow imaging

The *in vivo* bio-imaging experiments were carried out using Hepa1-6 tumor-bearing mice (C57BL/6) anesthetized with chloral hydrate (200 µL, 4%) in advance. Then 100 µL 10-min pre-irradiated ZGGO:Cr,Bi@mSiO₂-ZnPc aqueous solution (5 mg mL⁻¹) using 635 nm laser was administrated into mouse through the intratumoral injection. Furthermore 200 µL 10-min pre-irradiated ZGGO:Cr,Bi@mSiO₂-ZnPc aqueous solution (1 mg mL⁻¹) using 635 nm laser was administrated into tumor-bearing mouse through a tail vein injection. In two cases, *in vivo* deep red afterglow bioimaging were recorded at different

detecting time using Berthold NightOWL LB 983 equipment (emission filter: 700 nm; exposure time: 200 s) without external excitation source.

2.10. *In vivo* photodynamic therapy assay

Six groups ($n = 4$ per group) of Hepa1-6 cells tumor-bearing mice (C57BL/6), which tumor sizes were $\sim 32 \text{ mm}^3$, were prepared for *in vivo* PDT assay. Mice in different groups were injected different solutions (50 μL per mouse) by an intratumoral injection. The solutions corresponding to the six groups are PBS (I), ZGGO:Cr,Bi@mSiO₂-ZnPc solution (10 mg mL^{-1}) (II), free ZnPc (III), 10-min pre-irradiated ZGGO:Cr@mSiO₂-ZnPc solution (10 mg mL^{-1}) (IV), 10-min pre-irradiated ZGGO:Cr,Bi@mSiO₂-ZnPc solution (10 mg mL^{-1}) (V) and 10-min pre-irradiated ZGGO:Cr,Bi@mSiO₂-ZnPc solution (10 mg mL^{-1}) (VI), respectively. In the cases of group III, the tumor area was irradiated for 10 min using 635 nm laser (150 mW cm^{-2}) after the injection. In the cases of group VI, the tumor area was irradiated for 10 min using 635 nm laser (150 mW cm^{-2}) at 3 h after the injection. The PDT effect was evaluated by monitoring the changes in tumor volume and body weight of mice in each group every day up to 14 days. The tumor volume was calculated by the following Eq. (3):

$$V = L \times W^2 \times 1/2 \quad (3)$$

where L and W are the longest and shortest diameters (mm) of the tumor, respectively. The relative tumor volume was acquired by the ratio of the final volume to the initial volume of the tumor of each mouse. Suppression ratio (R_s) of tumor growth was calculated by following Eq. (4) [48]:

$$R_s = (V_c - V_t)/V_c \times 100\% \quad (4)$$

where V_t and V_c represent the mean tumor volume of the control group and treatment groups, respectively. All *in vivo* experimental sections are in conformity with the Chinese Animal Welfare Law and have been approved by the Ethics Committee on Laboratory Animal Care and Use of Northeast Normal University.

2.11. *In vivo* biosafety analysis

Healthy male C57BL/6 mice ($n = 4$ per group) were injected 200 μL ZGGO:Cr,Bi@mSiO₂-ZnPc aqueous solution (1 mg mL^{-1}). After 14-day raising, mice blood samples were collected for blood chemistry tests. The mice treated with PBS were used as the blank control.

3. Results and discussion

3.1. The enhanced deep red afterglow emissions of ZGGO:Cr³⁺,Bi³⁺ NPs

To obtain the sufficient afterglow intensity of ZGGO:Cr³⁺ nanoparticles, a Bi³⁺ substitution engineering was implemented in the synthesis of ZGGO:Cr³⁺ nanoparticles. The ZGGO:Cr³⁺,Bi³⁺ DRPLNPs were prepared by a modified sol-gel method in combination with a following vacuum-heat treatment (at 800 °C). The crystallographic and morphological properties of these ZGGO:Cr³⁺,Bi³⁺ NPs were characterized in the supporting information (combined with Fig. S1, supporting information). All the diffraction peaks can be well matched with both the standard Zn₂GeO₄ phase (JCPDS File: 25-1018, an anti-spinel crystal structure) and ZnGa₂O₄ phase (JCPDS File 38-1024, spinel crystal structure), indicating that ZGGO host might be the so-called solid-state solution of Zn₂GeO₄ and ZnGa₂O₄ [31,33,49,50]. For these ZGGO:Cr³⁺,Bi³⁺ DRPLNPs, excitation and emission (normalized) spectra related to Cr³⁺ in an intermediate crystal field were obtained as shown in Fig. S2 (supporting information). Strikingly, upon excitation of the red band, e.g. 592 nm, the emissions consist of a narrow peak $\sim 696 \text{ nm}$ and a broad band centered around 712 nm, which can be assigned to the ${}^2\text{E}$, ${}^4\text{T}_2({}^4\text{F}) \rightarrow {}^4\text{A}_2$ transitions of Cr³⁺ ions, respectively. The relationship between spectra of Cr³⁺ ions and the local crystal field strength were discussed in supporting information. After stopping 5 min irradiation by 635 nm laser, afterglows of ZGGO:Cr³⁺,Bi³⁺ nanoparticles monitored at 696 nm ($\lambda_{\text{mon}} = 696 \text{ nm}$) are recorded (Fig. 1a), where the afterglow intensity increases with increasing Bi

Fig. 1. (a) Afterglow decay curves of ZGGO:Cr_{0.02}Bi_x nanoparticles ($x = 0, 0.04, 0.08, 0.12$) (monitored at 696 nm after stopping 5 min irradiation with 635 nm laser). The inset in Fig. 1a shows phosphorescence spectra at 900 min after stopping 635 nm laser irradiation. (b) Deep red afterglow images of ZGGO:Cr_{0.02}Bi_x pellets ($x = 0, 0.12$) taken under white light and at different times after stopping 5 min irradiation of 635 nm laser at room temperature using a digital camera. Exposure time: 0.1 s (white light irradiation), 30 s (635 nm laser pre-irradiation). ISO: 200 (white light irradiation), 6400 (635 nm laser pre-irradiation). Camera model: Nikon D750. (c) A schematic diagram of deep red persistent luminescence of ZGGO:Cr³⁺ DRPLNPs related to the thermal activation process and/or the tunneling processes under red light excitation. The silver-gray balls and the red circle stand for the excited electrons and the generated holes, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

concentration and their afterglow times exceed 900 min. The deep red afterglow spectra at ~ 900 min are given in the inset of Fig. 1a. For the sample containing Bi ($x = 0.12$), the afterglow intensity increases to 4 times compared to the sample without Bi. This can be attributed to the facilitated tunneling process and the increase numbers and depths of deep traps induced by Bi substitution as demonstrated in Fig. S3 and the related supporting information. In addition, this enhanced deep red afterglow is also visualized from the pellet images of ZGGO:Cr_{0.02}Bi_x samples ($x = 0, 0.12$) after stopping 635 nm light irradiation (Fig. 1b). Strikingly, 635 nm light near NIR-I bio-window has relatively deep penetration depth in tissue and less harmful for normal cells.

To illustrate the enhancement mechanism for deep red persistent luminescence of ZGGO:Cr³⁺ DRPLNPs after doping Bi³⁺, a schematic for the energy levels of Cr³⁺ ion and trap levels with respect to the conduction and valence bands of ZGGO host is shown in Fig. 1c. In the case of red light (635 nm laser) irradiation, the electron can only be excited to the ⁴T₂(⁴F) level of Cr³⁺, and can be trapped by the deep traps through tunneling processes. Meanwhile the related thermal activation processes are blocked, i.e. the excited electron (e⁻) cannot be thermally activated to the conduction band and captured by the traps, because the larger energy difference between ⁴T₂(⁴F) level and

conduction band. Here, the deep traps relates to the tunneling process might be linked to the anti-site defect (Ga_{Zn}[•]-Zn_{Ga}[•]) in ZGGO:Cr³⁺ DRPLNPs, close to the distorted Cr³⁺ ions [33,51]. In addition, doped Bi³⁺ can also serve as electron traps, i.e. Bi³⁺ captures e⁻ and they constitute a complex of “Bi³⁺ + e⁻”, and can facilitate the tunneling processes from Cr³⁺ to deep traps and can enhance deep red emissions. The appearance of Bi with lower valence state was validated by analyzing XPS spectra as shown in Fig. S4a and Table S1 (supporting information). The amount of Bi with lower oxidation state increases with increasing Bi concentration, indicating that more Bi³⁺ ions act as deep traps in ZGGO:Cr³⁺ nanoparticles with a larger amount of Bi, which were detailedly demonstrated in supporting information related to Figs. S4b, S5 and Table S2. To further confirm the occurred tunneling process from Cr³⁺ to deep traps related to Bi in the case of 635 nm excitation, the increasing luminescence and afterglow intensity with increasing the excitation time under 635 nm laser excitation can be observed as shown in Fig. S6a and b, respectively. Especially, the luminescent intensity increases rapidly with increasing excitation time in the beginning of 635 nm laser on. While the excitation time is longer than ~ 1 min, the excitation-time-dependent luminescent intensity exhibits a slow rise tendency. These results indicate that the traps in ZGGO:Cr_{0.02}Bi_{0.12}

Fig. 2. (a) XRD patterns and (b, c) TEM photographs afterglow decay curves of ZGGO:Cr_{0.02} and ZGGO:Cr_{0.02}Bi_{0.12} nanoparticles annealed at 750 °C in vacuum. (d) EDX spectrum of ZGGO:Cr_{0.02}Bi_{0.12} LT-DRPLNPs. The inset shows the weight and atomic percentage of the host elements (Zn, Ga, Ge and O). (e) Afterglow decay curves of ZGGO:Cr_{0.02} and ZGGO:Cr_{0.02}Bi_{0.12} in panel b and c. Before recording the afterglow decay curves ($\lambda_{\text{mon}} = 696$ nm), all the samples were irradiated for 5 min with 635 nm laser. The upper inset shows the phosphorescence spectra at 900 min after stopping 635 nm light irradiation.

Fig. 3. (a) Deep red afterglow images of ZGGO:Cr_{0.02},Bi_{0.12} pellet covered by 5-mm thick pork tissue taken at different decay times in the cases of the nth and n + 1th red light (635 nm) irradiations in dark field (the upper panel) and merged with the cover tissue (the lower panel). Exposure time: 30 s. ISO: 6400. Digital camera model: Nikon D750. (b) The afterglow decay curves of ZGGO:Cr_{0.02},Bi_{0.12} pellet covered by 5-mm thick pork tissue in the cases of the nth and n + 1th red light (635 nm) irradiations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nanoparticles can be charged within 10 min through a tunneling process. In addition, XPS spectra of Bi 4f_{7/2} and 4f_{5/2} core levels of ZGGO:Cr_{0.02},Bi_{0.12} with different pre-irradiation time under 635 nm laser excitation (150 mW cm⁻²) and the related fitting results are shown in Fig. S6c and Table S3, respectively. These results suggest that Bi³⁺ ions can serve as electron traps, i.e. Bi³⁺ captures an electron (e⁻) and they constitute a complex of “Bi³⁺ + e⁻”. More details can be found in supporting information.

3.2. Low-temperature-synthesized ZGGO:Cr,Bi LT-DRPLNPs and deep red afterglow bioimaging

For the past few years, the enhanced permeability and retention (EPR) effect has been regarded as a key pharmacokinetic feature of nanomedicines and it leads to the facilitated passive accumulation of nano-sized molecules or particles in tumor tissues and the reduced toxicity in normal tissues [52–54]. To realize bioimaging and therapy utilizing EPR effect, the size distribution of nanoplateforms or particles should be in the range of 20–200 nm as the reported studies [53,55]. In particular, nanomedicines in the sub-100 nm range have exhibited higher penetration and accumulation in tumor tissues with hypovascular and hypopermeable environments [56]. However, for ZGGO:Cr_{0.02},Bi_x (x = 0.04, 0.08, 0.12) DRPLNPs annealed at 800 °C, their size distributions are in the ranges of 30–180 nm, 60–180 nm and 70–350 nm, respectively. There are many particles with larger diameters than 100 nm in these samples.

To meet the requirements of afterglow imaging and penetration and accumulation in tumor tissues, ZGGO:Cr_{0.02},Bi_{0.12} powders were synthesized successfully by the modified sol-gel method in combination with a following vacuum-heat treatment at lower temperature (at 750 °C) and named as ZGGO:Cr_{0.02},Bi_{0.12} LT-DRPLNPs. The XRD patterns of the ZGGO:Cr_{0.02},Bi_x (x = 0, 0.12) LT-DRPLNP are shown in

Fig. 2a. All the diffraction peaks can be well matched with both the standard Zn₂GeO₄ phase (JCPDS File: 25-1018) and ZnGa₂O₄ phase (JCPDS File 38-1024), indicating that ZGGO:Cr_{0.02},Bi_x might be the so-called solid-state solution of Zn₂GeO₄ and ZnGa₂O₄ [31,33,49,50]. To further confirm the crystal structure of ZGGO LT-DRPLNPs, Rietveld refinement was performed as reported by Xiong et al. [57,58]. The XRD Rietveld refinement for ZGGO:Cr_{0.02},Bi_{0.12} LT-DRPLNPs is shown in Fig. S7. It can be found that the crystal structure data of standard ZnGa₂O₄ phase (JCPDS File 38-1024, cubic Fd-3 m space group) were very well used as the starting model for structure solution. The calculated mole ratio of Zn:Ga:Ge (8:12:1:3.4) is very close to 8:12:3 (i.e. nominal stoichiometric mole ratio: Zn:Ga:Ge = 2:2.98:0.75). In particular, the values of R_p and R_{wp} were 6.6% and 9.6%, respectively. This satisfying result suggests that ZGGO LT-DRPLNPs can be assigned to ZnGa₂O₄ phase (JCPDS File 38-1024). From the TEM micrographs shown in Fig. 2b and 2c, the average particle sizes of the ZGGO:Cr_{0.02},Bi_x samples with and without Bi (x = 0.12, 0) are ~58 and ~52 nm, respectively, and the size distributions are shown in Fig. S8a and b (supporting information). Meanwhile, to investigate the chemical formula of ZGGO sample, EDX spectrum of ZGGO:Cr_{0.02},Bi_{0.12} is shown in Fig. 2d. The calculated weight and atomic percentage of the host elements (Zn, Ga, Ge and O) is given in the inset of Fig. 2d. For our ZGGO host, the obtained mole ratio of Zn:Ga:Ge (10:10:3) is very close to 8:12:3 (i.e. nominal stoichiometric mole ratio: Zn:Ga:Ge = 2:2.98:0.75). This result suggests that the so-called solid-state solution of Zn₂GeO₄ and ZnGa₂O₄ might be formed. Afterglow decay curves (λ_{mon} = 696 nm) of ZGGO:Cr_{0.02},Bi_x nanoparticles (x = 0, 0.12) are shown in Fig. 2e, where the afterglow is also enhanced by Bi substitution. The afterglow spectra at ~900 min are given in the inset of Fig. 2e. In the following, the ZGGO:Cr_{0.02},Bi_{0.12} LT-DRPLNPs (synthesized at 750 °C) will be employed for irradiation-free PDT study subject to biocompatibility treatment.

To realize long-term deep red afterglow imaging guidance, a pellet pressed by the ZGGO:Cr_{0.02},Bi_{0.12} nanoparticles annealed at 750 °C in vacuum was used as an object (represent as LT-DRPLNPs pellet). In front of a digital camera, LT-DRPLNPs pellet was covered by a 5-mm thick pork tissue. In fact, a deep red afterglow imaging can be acquired at any time after stopping red light (635 nm) re-irradiation. For example, Fig. 3a shows the nth and n + 1th deep red afterglow images of LT-DRPLNPs pellet taken at different times (5, 10 min) after stopping 10-min irradiation with a 635 nm CW laser (150 mW cm⁻²) in the dark field. Here the time interval between the nth and n + 1th imaging is 120 min. For the imaging taken at the same decay time after stopping 635 nm irradiation, the quality of the nth imaging is equal to that of the n + 1th imaging. Meanwhile, after a merging operation on the images and tissues, the pork tissue textures are well tallied with the deep red images. In particular, in the cases of the nth and n + 1th 635 nm irradiations, the almost same deep red afterglow decay curves were obtained as shown in Fig. 3b, further suggesting high-quality deep red afterglow bioimaging can be realized by an intermittent red light re-irradiation strategy.

3.3. Surface functionalization of ZGGO:Cr,Bi nanoparticles and in vitro PDT

For the aforementioned irradiation-free PDT, cancer cell were killed by ¹O₂ molecules through two different generated paths as shown in Fig. 4a, i.e. the loaded ZnPc molecules were excited by 635 nm light and the deep red emissions of ZGGO:Cr,Bi cores upon 635 nm excitation (path 1) *in vivo* and by the deep red afterglow emissions (path 2) after stopping irradiation *in vitro* and/or *in vivo*, respectively. The corresponding PDT process can be monitoring by deep red afterglow imaging. To obtain this irradiation-free PDT nanoplateform, ZnPc molecules were loaded into mesoporous silica coated on the surface of ZGGO:Cr,Bi LT-DRPLNP. The surface modification of ZGGO:Cr_{0.02},Bi_{0.12} LT-DRPLNP was executed in two steps, i.e. an ultra-thin (1–3 nm)

Fig. 4. (a) A schematic illustration of the surface functionalization of ZGGO:Cr,Bi nanoparticles and irradiation-free PDT. TEM images of (b) amorphous SiO₂ coated ZGGO:Cr,Bi and (c) ZGGO:Cr,Bi@mSiO₂ nanoplateforms. (d) Fourier transform infrared transmittance spectra of ZGGO:Cr,Bi and ZGGO:Cr,Bi@mSiO₂ nanoplateforms. (e) Absorption spectrum of ZnPC photosensitizers in DMSO and the afterglow spectrum of ZGGO:Cr,Bi@mSiO₂ nanoplateforms. The inset shows a photo of ZnPC-loaded ZGGO:Cr,Bi@mSiO₂ (ZGGO:Cr,Bi@mSiO₂-ZnPc) nanoplateforms. (f) Afterglow decay curve of ZGGO:Cr,Bi@mSiO₂-ZnPc/HSA solution (5 mg mL⁻¹) after stopping the 10-min irradiation with 635 nm laser. The right upper inset shows the phosphorescence at 60 min after stopping 635 nm irradiation. The middle insets show the images of the ZGGO:Cr,Bi@mSiO₂-ZnPc/HSA solution taken using a digital camera (Model: Nikon D750) under white light (Exposure time: 0.1 s, ISO: 200), 635 nm CW laser irradiations (Exposure time: 0.1, ISO: 200) and at 30 s after stopping 10-min irradiation with a 635 nm laser in the dark field (exposure time: 30 s, ISO: 6400).

amorphous SiO₂ shell and a ~15 nm thick mesoporous silica shell was coated on the surface of LT-DRPLNPs in sequence via a Stöber approach shown in Fig. 4b and c. Fourier transform infrared spectra of ZGGO:Cr,Bi nanoparticles and ZGGO:Cr,Bi@mSiO₂ nanoplateforms were measured and are shown in Fig. 4d. For the ZGGO:Cr,Bi@mSiO₂ nanoplateforms, new absorption bands appear in relation with Si-O-Si (1080 cm⁻¹, 803 cm⁻¹) and Si-OH (951 cm⁻¹) appear compared to the ZGGO:Cr,Bi bare core, supporting the formation of the core/shell structure [59]. The phosphorescence spectrum of ZGGO:Cr,Bi@mSiO₂ nanoplateforms is partly overlapped with absorption of ZnPC molecule, as shown in Fig. 4e. Thus after loading ZnPC molecules and stopping 635 nm irradiation, deep red afterglow is the only responsible for the possible generation of ¹O₂ molecules. The corresponding color change of samples before and after loading is also shown in the inset. The amount of ZnPC loaded into the pores of ZGGO:Cr,Bi@mSiO₂ nanoplateforms was determined using the method as reported by Idris et al.

[8,60]. The absorption spectra of the ZnPC solutions without ZGGO:Cr,Bi@mSiO₂ nanoplateforms before and after loading ZnPC into these nanoplateforms and those of the standard ZnPC solutions (DMSO solvent) with various ZnPC concentrations were measured and are shown in Fig. S9a and b, respectively. The standard concentration curve of ZnPC solutions (DMSO solvent) was obtained by fitting the concentration-dependent absorbance data of the aforementioned standard ZnPC solution at 672 nm as shown in Fig. S10. The absorbance difference between the absorption peaks at 672 nm of ZnPC solutions without ZGGO:Cr,Bi@mSiO₂ nanoplateforms before and after loading ZnPC into these nanoplateforms is ~0.2083. Thus the ZnPC loading efficiency (LE) is found to be 0.346 wt%.

In addition, for the ZGGO:Cr,Bi@mSiO₂-ZnPc nanoplateforms dispersed in a human serum albumin solution (HSA, 5 mg mL⁻¹) after 635 nm irradiation (irradiation time: 10 min), the afterglow time exceeds 60 min, as shown in Fig. 4f. This result is supported by the

observed phosphorescence of the ZGGO:Cr,Bi@mSiO₂-ZnPc/HSA solution as shown in the upper right inset of Fig. 4f. The clear images of the ZGGO:Cr,Bi@mSiO₂-ZnPc/HSA solution were taken under white light, 635 nm CW laser irradiations and at 30 s after stopping 10-min irradiation with a 635 nm laser in the dark field, as shown in the middle insets of Fig. 4f, suggest that the afterglow tracing of PDT is likely. Meanwhile the stability of ZGGO:Cr,Bi@mSiO₂-ZnPc nanoplateforms dispersed in various media, such as H₂O, normal saline, PBS, HSA and DMEM, was evaluated by measuring photoluminescence spectra monitored at 696 nm of ZGGO:Cr,Bi@mSiO₂-ZnPc solutions under 635 nm excitation after letting them stand for different time (0, 1, 3, 6, 12, 24, 48 and 168 h) in dark at 4 °C as shown in Fig. S11a–e. For each solution, the similar spectrum and nearly equal intensity at different time points suggest that ZGGO:Cr,Bi@mSiO₂-ZnPc nanoplateforms dispersed in different media has a good stability.

To demonstrate *in vivo* bioimaging using deep red afterglow of the related nanoplateforms, 100 μ L ZGGO:Cr,Bi@mSiO₂-ZnPc solution (5 mg mL⁻¹) was administrated into a Hepa 1–6 tumor-bearing C57BL/6 mouse through an intratumoral injection. Before the injection, 635 nm laser (150mW cm⁻²) irradiated the related solution for 10 min. The clear deep red afterglow images of ZGGO:Cr,Bi@mSiO₂-ZnPc nanoplateforms existing in the injected area of the mouse can be acquired when the detecting time increases from 5 to 240 min as shown in Fig. 5a (exposure time: 200 s). Furthermore 200 μ L ZGGO:Cr,Bi@mSiO₂-ZnPc aqueous solution (1 mg mL⁻¹) with 10-min 635 nm laser pre-irradiation was injected into another tumor-bearing C57BL/6 mouse via tail vein. The clear deep red afterglow images of the tumor area can be detected in tumor area till 180 min after the injection as shown in Fig. 5b, suggesting that ZGGO:Cr,Bi@mSiO₂-ZnPc nanoplateforms can be accumulated into the tumor area of the tumor-bearing mouse. To better trace the accumulation of the related nanoplateforms into tumor area, the tumor area of the tumor-bearing mouse was re-irradiation for 10 min using 635 nm laser at 180 min after tail vein injection. The clearer deep red afterglow image of the tumor area can be observed compared to that before the re-irradiation. Moreover, after the tail vein injection, the accumulations of ZGGO:Cr,Bi@mSiO₂-ZnPc

nanoplateforms into other organs were found as shown in Fig. 5b. After we dissected the tumor and major organs of tumor-bearing C57BL/6 mouse, strong deep red afterglow signals can be detected from the organs of heart, liver kidney and tumor, respectively, as shown in Fig. 5c. These results suggest that the deep red afterglow can be used for tracing the PDT.

To evaluate the ¹O₂ quantum yield of the ZGGO:Cr,Bi@mSiO₂-ZnPc nanoplateforms via two paths, 1,3-diphenylisobenzofuran (DPBF) and methylene blue (MB) are used as an acceptor of ¹O₂ and a reference reagent, respectively. Because DPBF will be continuously consumed by the generated ¹O₂, the decrease in the absorbance of DPBF with increasing the amount of ¹O₂ can provide a way to measure the ¹O₂ generation rate as reported by Jia et al. [46]. In this study, for the sake of calibration, the absorbance of ZGGO:Cr,Bi@mSiO₂-ZnPc and MB in EtOH (ethyl alcohol) excited by 635 nm CW laser was adjusted to ~0.065 OD through modulating solute-solvent mass ratio, as shown in Fig. S12 (Supporting Information). Then 15 μ L DPBF solution (2 mg mL⁻¹) was added into the aforementioned ZGGO:Cr,Bi@mSiO₂-ZnPc/EtOH and MB/EtOH solutions in dark place, respectively. Under 635 nm laser (50 mW cm⁻²) irradiation, the absorption spectra of DPBF ($\lambda_{\text{max}} = 411$ nm) dispersed in the measured ZGGO:Cr,Bi@mSiO₂-ZnPc/EtOH and MB/EtOH solutions as a function of irradiation time are shown in Fig. 6a and Fig. S13 (Supporting Information), respectively. It can be found that the absorbance of DPBF decreases with increasing irradiation time, suggesting the generation of ¹O₂ molecules. Meanwhile, based on the irradiation-time dependent absorbance of DPBF ($\lambda_{\text{max}} = 411$ nm) in the solutions of ZGGO:Cr,Bi@mSiO₂-ZnPc/EtOH and MB/EtOH as shown in Fig. 6b, the ¹O₂ quantum yield of the ZGGO:Cr,Bi@mSiO₂-ZnPc nanoplateforms via path1 was calculated to be ~0.396. Here, ¹O₂ quantum yield (0.49) of MB in EtOH is used as a standard [46]. The detail related to the calculation is described in the experiment section later. In our case, the obtained ¹O₂ quantum yield is larger than that using the upconversion luminescence strategy reported by Idris et al (0.364) [8]. The larger ¹O₂ quantum yield might be related to the fact that the ¹O₂ can be generated under 635 nm laser excitation, which includes the contribution from ZnPc molecules excited by

Fig. 5. (a) *In vivo* deep red afterglow images of Hepa 1–6 tumor-bearing C57BL/6 mouse acquired at different detecting time after intratumoral injection of 100 μ L ZGGO:Cr,Bi@mSiO₂-ZnPc solution (5 mg mL⁻¹) with 635 nm laser pre-irradiation for 10 min. (b) *In vivo* deep red afterglow images of tumor-bearing C57BL/6 mouse acquired at different detecting times after tail vein injection of 200 μ L ZGGO:Cr,Bi@mSiO₂-ZnPc aqueous solution (1 mg mL⁻¹) with 10 min 635 nm laser pre-irradiation and deep red afterglow image of the tumor-bearing mouse acquired at 180 min after the injection through 635 nm laser re-irradiation for 10 min. (c) Deep red afterglow images of tumor and major organs dissected from the tumor-bearing mouse detected at 180 min after the tail vein injection. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. (a) Absorption spectra of DPBF in the calibrated ZGGO:Cr,Bi@mSiO₂ and MB EtOH solution as a function of irradiating time under 635 nm laser (50 mW cm⁻²) irradiation. (b) The irradiation-time dependent absorbance of DPBF (λ_{max} = 411 nm) dispersed in the calibrated ZGGO:Cr,Bi@mSiO₂ and MB EtOH solutions and their fitting data (red lines). (c) Absorption spectra of the DPBF dispersed in the 635 nm pre-irradiated ZGGO:Cr,Bi@mSiO₂-ZnPc/EtOH solution as a function of decay time. (d) Decay curves of the absorbance (λ_{max} = 411 nm) of the DPBF dispersed in the 635 nm pre-irradiated ZGGO:Cr,Bi@mSiO₂-ZnPc/EtOH solution and its fitting data (red line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

635 nm lights and the deep red emissions of ZGGO:Cr,Bi cores under 635 nm excitation (path 1). To further confirm the ¹O₂ generation induced by deep red afterglow emissions (path 2), the absorption spectra of the DPBF dispersed in the 635 nm pre-irradiated ZGGO:Cr,Bi@mSiO₂-ZnPc/EtOH solution as a function of decay time are shown in Fig. 6c and 6d. It can be found that the decrease in the absorbance (λ_{max} = 411 nm) with decay time of deep red afterglow emissions suggests that the ¹O₂ molecules are generated by the excitation of ZnPc under deep red afterglow emissions. According to the aforementioned method, the ¹O₂ quantum yield induced by deep red afterglow emissions is ~ 0.038, which is about one-tenth of the total yield in the case of irradiation with 635 nm laser.

To assess how the surface functionalization of ZGGO:Cr,Bi LT-DRPLNPs influences the biocompatibility, a standard methyl thiazolyl tetrazolium (MTT) assay was administrated on Hepa 1-6 (Hepatoma 1-6) cancer cells and the cell viabilities exposed to ZGGO:Cr,Bi LT-DRPLNPs, ZGGO:Cr,Bi@mSiO₂ and ZGGO:Cr,Bi@mSiO₂-ZnPc nanoplateforms are shown in Fig. 7a. Compared to the control group incubated with PBS, it can be found that the viability decreases from 100 to 60% with increasing ZGGO:Cr,Bi concentration, suggesting ZGGO:Cr,Bi nanoparticles possess intrinsic cytotoxicity. The intrinsic cytotoxicity might be related to the heavy metal ions on the surface of naked ZGGO:Cr,Bi nanoparticles [61]. For the nanoplateforms coated by mesoporous silica, the cell viabilities are improved to more than 80% in

the concentration range of 0–100 μg mL⁻¹. To demonstrate the effect of ZnPc loading on the cell viability, the viabilities of the Hepa 1-6 cells as a function of ZGGO:Cr,Bi@mSiO₂-ZnPc concentration in the cases of irradiation with and without 635 nm laser are shown in Fig. 7b. In the aforementioned MTT experiment, Hepa1-6 cells were preincubated for 24 h with various concentrations of ZGGO:Cr,Bi@mSiO₂-ZnPc nanoplateforms. After the incubated Hepa 1-6 cells were irradiated for 10 min by 635 nm laser, they were cultured for 4 h again. In the case of irradiation with 635 nm laser, the cell viability decreases from 100 to 20% with increasing ZGGO:Cr,Bi@mSiO₂-ZnPc concentration from 0 to 100 μg mL⁻¹, suggesting the cells were killed by the ¹O₂ generated through path 1 and 2, i.e. the contributions from ZnPc molecules excited by 635 nm light and the deep red emissions of ZGGO:Cr,Bi cores upon 635 nm excitation. This result is further supported by confocal laser scanning microscope images of calcein-AM/propidium iodide (PI) co-stained Hepa 1-6 cells incubated with PBS (control), ZGGO:Cr,Bi, ZGGO:Cr,Bi@mSiO₂ and ZGGO:Cr,Bi@mSiO₂-ZnPc in the cases of irradiation with and without 635 nm laser (irradiation time: 5 min, irradiation power density: 150 mW cm⁻²) as shown in Fig. 7c. Here viable cells were stained green with Calcein-AM, and dead cells were stained red with PI. For control group, in the cases with and without 635 nm laser irradiations, the similar viabilities of the cells suggest that the use of the red light (635 nm) will not do harm to normal cells. In the case without 635 nm laser irradiation, for the groups treated by mSiO₂

Fig. 7. (a) Cell viabilities of Hepa 1–6 cells incubated with PBS (control), ZGGO:Cr,Bi@mSiO₂ and ZnPc-loaded ZGGO:Cr,Bi@mSiO₂ measured by MTT assay. (b) The viabilities of the Hepa 1–6 cells as a function of ZGGO:Cr,Bi@mSiO₂-ZnPc concentration in the cases with and without 635 nm laser irradiations. (c) Confocal laser scanning microscope images of Calcein-AM/PI co-stained Hepa 1–6 cells incubated with PBS (control), ZGGO:Cr,Bi, ZGGO:Cr,Bi@mSiO₂ and ZGGO:Cr,Bi@mSiO₂-ZnPc in the cases with and without 635 nm laser irradiations (irradiation time: 5 min, irradiation power density: 150 mW cm⁻²). Viable cells are stained green with Calcein-AM and dead cells are stained red with PI. Scale bar is 200 μm . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

coated ZGGO:Cr,Bi nanoplateforms (ZGGO:Cr,Bi@mSiO₂ and ZGGO:Cr,Bi@mSiO₂-ZnPc), cell survival rates are higher than that treated by the ZGGO:Cr,Bi nanoparticles. For the Hepa 1–6 cells treated with ZGGO:Cr,Bi@mSiO₂-ZnPc, 635 nm laser irradiation leads to the appearance of more dead Hepa 1–6 cells due to the ¹O₂ generation. This result is consistent with their MTT assay analysis. The merged images suggest that the Hepa 1–6 cells almost were killed during the 5 min irradiation with 635 nm laser. Because the MTT assay was measured at 4 h after stopping 635 nm irradiation, the dead cells are attributed to the ¹O₂ generation through path 1 and 2.

To further evaluate the intracellular efficacy of ROS induced by deep red afterglow of ZGGO:Cr,Bi@mSiO₂-ZnPc (path 2), DCFH-DA was selected as the fluorescence probe of generated ROS in cells, as reported by Wang et al. [62]. DCFH-DA can be oxidized into 2',7'-

dichlorofluorescein (DCF) by ROS [63]. The DCF has green emission under 488 nm excitation. Detection of intracellular ROS generation in Hepa 1–6 cells treated for 24 h by PBS (control) and by ZGGO:Cr,Bi@mSiO₂-ZnPc solutions (100 $\mu\text{g mL}^{-1}$) without and with subsequent 10-min 635 nm irradiation (150 mW cm⁻²) are shown in Fig. 8. Here DCFH-DA was loaded into the aforementioned treated Hepa1-6 cells with a 30-min incubation without additional external irradiation. For the cells treated with PBS and with ZGGO:Cr,Bi@mSiO₂-ZnPc solution without 635 nm irradiation, green fluorescence signals cannot be observed inside cells under 488 nm excitation, suggesting that ROS were not generated. For the cells treated with ZGGO:Cr,Bi@mSiO₂-ZnPc solution with 635 nm irradiation, green fluorescence signals can be clearly acquired inside the cells. This result suggests that ROS were generated inside the cells under deep red afterglow (696 nm)

Fig. 8. Detection of intracellular ROS generation in Hepa 1–6 cells treated for 24 h by PBS (control) and by ZGGO:Cr,Bi@mSiO₂-ZnPc solution (100 $\mu\text{g mL}^{-1}$) solutions without and with subsequent 10-min 635 nm laser irradiation (150 mW cm^{-2}). DCFH-DA was selected as the fluorescence probe of ROS inside cells. All the scale bars are 50 μm .

excitation.

3.4. *In vitro* and *in vivo* irradiation-free PDT induced by deep red afterglow emissions

To demonstrate *in vitro* irradiation-free PDT induced by enhanced deep red afterglow, the confocal laser scanning microscope images of Calcein-AM/PI co-stained Hepa 1–6 cells pre-incubated for 12 h with PBS (control), 10-min pre-irradiated ZGGO:Cr@mSiO₂-ZnPc and 10-min pre-irradiated ZGGO:Cr,Bi@mSiO₂-ZnPc (irradiation wavelength: 635 nm, irradiation power density: 150 mW cm^{-2}) are shown in Fig. 9a. Compared with control group, very few dead cells can be found in the group treated with pre-irradiated ZGGO:Cr@mSiO₂-ZnPc solution, suggesting that the weak deep red afterglow of the related nano-platform without Bi cannot generate enough ¹O₂. However, for the group treated by pre-irradiated ZGGO:Cr,Bi@mSiO₂-ZnPc solution, almost all of cells were killed due to the generation of more ¹O₂ induced by the enhanced deep red afterglow after Bi doping. To further confirm this inference, cell viabilities of Hepa 1–6 cells incubated with PBS

(control), pre-irradiated ZGGO:Cr@mSiO₂-ZnPc solution and pre-irradiated ZGGO:Cr,Bi@mSiO₂-ZnPc solution measured by a standard MTT assay are shown Fig. 9b. It can be found that the viability of cells treated with pre-irradiated ZGGO:Cr@mSiO₂-ZnPc solution is higher than that of cells treated with the solution containing Bi (pre-irradiated ZGGO:Cr,Bi@mSiO₂-ZnPc) at different solution concentrations. In particular, when the solution concentration is 100 $\mu\text{g mL}^{-1}$, their viability difference was increased to $\sim 70\%$, indicating more ¹O₂ molecules were generated due to the enhanced deep red afterglow after Bi doping.

To demonstrate the effect of *in vivo* irradiation-free PDT, the tumor-bearing mice (C57BL/6) were randomly divided into six groups ($n = 4$ per group) treated under different conditions. Mice in different groups were injected different solutions (50 μL per mouse) by an intratumoral injection. The solutions corresponding to the six groups are PBS (I), ZGGO:Cr,Bi@mSiO₂-ZnPc solution (10 mg mL^{-1}) (II), free ZnPc (III), 10-min pre-irradiated ZGGO:Cr@mSiO₂-ZnPc solution (10 mg mL^{-1}) (IV), 10-min pre-irradiated ZGGO:Cr,Bi@mSiO₂-ZnPc solution (10 mg mL^{-1}) (V) and 10-min pre-irradiated ZGGO:Cr,Bi@mSiO₂-ZnPc solution (10 mg mL^{-1}) (VI), respectively. In the cases of group III, the

Fig. 9. (a) Cell morphologies (upper) and confocal laser scanning microscope images of Calcein-AM/PI co-stained Hepa 1–6 cells incubated with PBS (control), pre-irradiated ZGGO:Cr@mSiO₂-ZnPc and pre-irradiated ZGGO:Cr,Bi@mSiO₂-ZnPc. Irradiation wavelength: 635 nm. Irradiation time: 10-min. Irradiation power density: 150 mW cm⁻². All the scale bars are 50 μm . (b) Cell viabilities of Hepa 1–6 cells incubated with PBS (control), pre-irradiated ZGGO:Cr@mSiO₂-ZnPc and pre-irradiated ZGGO:Cr,Bi@mSiO₂-ZnPc measured by MTT assay. (c) The physical status (upper) and tumor photographs (bottom) of representative tumor-bearing C57BL/6 mice in six groups at two weeks after an intratumoral injection using various solutions. Solutions: PBS (I); ZGGO:Cr,Bi@mSiO₂-ZnPc solution (10 mg mL⁻¹) (II); free ZnPc (III); 10-min pre-irradiated ZGGO:Cr@mSiO₂-ZnPc solution (10 mg mL⁻¹) (IV); 10-min pre-irradiated ZGGO:Cr,Bi@mSiO₂-ZnPc solution (10 mg mL⁻¹) (V) 10-min pre-irradiated ZGGO:Cr,Bi@mSiO₂-ZnPc solution (10 mg mL⁻¹) (VI). In the cases of group III, the tumor area was irradiated for 10 min using 635 nm laser (150 mW cm⁻²) after the injection. In the cases of group VI, the tumor area was irradiated for 10 min using 635 nm laser (150 mW cm⁻²) at 3 h after the injection. (d) The relative tumor volume of different groups as a function of raising time after the intratumoral injection. Error bars were acquired from four mice per group. P values marked by asterisks were calculated based on Student's t-test (*P < 0.05). (e) All the tumor photographs of each mice per group.

tumor area was irradiated for 10 min using 635 nm laser (150 mW cm⁻²) after the injection. In the cases of group VI, the tumor area was irradiated for 10 min using 635 nm laser (150 mW cm⁻²) at 3 h after the injection. The physical status and tumor sizes of tumor-bearing C57BL/6 mice at two weeks after various treatments are shown in Fig. 9c. It can be found that the tumor size of the mouse in group I is close to that in group II. However, the tumor sizes of the mice in group III, IV, V and VI are smaller than those in group I and II, indicating that anticancer effect occurred due to ¹O₂ generation in the presences of ZnPc and deep red light excitation. Compared to group III, the smaller tumor size of the mouse in group V indicates that long term PDT induced by

strong deep red afterglow possesses better anticancer effect than short time PDT under external excitation. Meanwhile, for group IV, the tumor size is bigger than that in group V, indicating that the disappearance of Bi in the related nanoplateform leads to weak deep afterglow and the decreased number of ¹O₂. Importantly, for group VI, the smallest tumor size was observed among the six groups, suggesting that the growth of cancer cells was suppressed and the generation of more ¹O₂ molecules was induced by the enhanced deep red afterglow of the ZGGO:Cr,Bi@mSiO₂-ZnPc.

To further evaluate how deep red afterglow and 635 nm excitation influence *in vivo* PDT, the relative tumor volume of mice in six groups as

Fig. 10. Blood biochemistry test of mice treated with ZGGO:Cr,Bi@mSiO₂-ZnPc nanoplateforms. Six important hepatic and renal function indicators: (a) ALT; (b) ALB; (c) AST; (d) GLU; (e) BUN and (f) CRE. Mice treated with PBS used as the blank control.

a function of raising time after intratumoral injection were measured and are shown in Fig. 9d. For the groups I-IV, all the relative tumor volumes increase with increasing raising time after intratumoral injection. However the growth speed of tumor was decreased in the groups V and VI, suggesting that the strategy for generating ¹O₂ by deep red afterglow excitation can provide an irradiation-free PDT. Here, for group VI, the inhibition factor of cancer growth is ~80%. To further evaluate the physical status of six group mice, the mice body weights of tumor-bearing C57BL/6 mice as a function of raising time after the intratumoral injection using various solutions are shown in Fig. S14. Among the six groups, mice in group VI were the heaviest, suggesting the growth of tumor was suppressed. This result is in good agreement with the aforementioned analyses. Furthermore, all the tumor photographs acquired from six groups (n = 4 per group) are shown in Fig. 9e. It can be found that the tumor sizes in the groups V and VI were always the smallest among six groups, suggesting that *in vivo* irradiation-free PDT effect was reliable. In particular, in the case of group VI, the tumors of two mice disappeared. This result further demonstrates that the excellent anticancer effect of irradiation-free *in vivo* PDT based on the enhanced deep red afterglow of ZGGO:Cr,Bi@mSiO₂-ZnPc has potential application in biomedical field.

To evaluate the biosafety of ZGGO:Cr,Bi@mSiO₂-ZnPc nanoplateforms, blood biochemistry test examination was administrated on two groups of mice. One group was injected 200 μL PBS per mouse (blank control), the other group was injected 200 μL ZGGO:Cr,Bi@mSiO₂-ZnPc aqueous solution (1 mg mL⁻¹) per mouse. After 14-day raising, the mice blood samples were collected for blood chemistry tests. Six important hepatic and renal function indicators were examined, i.e. alanine aminotransferase (ALT), albumin (ALB), aspartate aminotransferase (AST), glucose (GLU), blood urea nitrogen (BUN) and creatinine (CRE). From the levels of these markers, no significant difference was observed between control and treatment groups as shown in Fig. 10, suggesting that ZGGO:Cr,Bi@mSiO₂-ZnPc nanoplateforms have excellent hepatic and renal safety profiles.

4. Conclusions

In summary, a new approach of Bi doping is introduced and validated to enhance the persistent luminescence of ZGGO:Cr DRPLNPs

with the afterglow time of more than 900 min. For the ZGGO:Cr_{0.02}Bi_{0.12} sample, the deep red afterglow intensity (~700 nm) is four times stronger than that without Bi. The relevant spectroscopy and chemical analyses indicate that this enhancement comes from the facilitated tunneling processes from the deep traps to the ⁴T₂ level of Cr³⁺ due to Bi substitution, where deep traps are formed by the doped Bi³⁺ ions. In particular, this enhancement makes it possible of the imaging-guided irradiation-free cancer therapy for the first time. Based on this robust afterglow LT-DRPLNPs, ZGGO:Cr,Bi@mSiO₂-ZnPc nanoplateforms were constructed for *in situ* imaging guided PDT without recovery of persistent luminescence. Proof of concept was performed for *in vitro* and *in vivo* models. The ¹O₂ quantum yield of 0.038 induced directly by the afterglow was reached. Two weeks after the intratumoral injection of 10 min pre-irradiated ZGGO:Cr,Bi@mSiO₂-ZnPc nanoplateforms, the inhibition factor of cancer growth was as high as ~80%. Our results suggest that autofluorescence-free ZGGO:Cr,Bi@mSiO₂-ZnPc nanoplateforms may have great potential for cancer treatment.

5. Data availability

The data is available to download from the website.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Additional analysis data and discussion, TSL, deep red afterglow curve, X-ray photoelectron spectroscopy, size distribution and the related descriptions. Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cej.2020.124067>.

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