Turning solid into gel for high-efficient persistent luminescence-sensitized photodynamic therapy

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\begin{abstract}
Bioavailable persistent luminescence material is an ideal internal light source for long-term photodynamic therapy, but inevitably suffers from low utilization efficiency and weak persistent luminescence due to corrosion and screening processes. Herein, we show a facile and smart “turning solid into gel” strategy to fabricate persistent luminescence hydrogel for high-efficient persistent luminescence-sensitized photodynamic therapy. The homogeneous persistent luminescence hydrogel was synthesized via dispersing high-temperature calcined persistent luminescence material without corrosion and screening into a biocompatible alginate-Ca\textsuperscript{2+} hydrogel. The simple synthesis strategy allows 100\% of utilization efficiency and intact persistent luminescence of persistent luminescence material. The persistent luminescence hydrogel possesses favorable biocompatibility, bright persistent luminescence, red light renewability, good syringeability, and strong fixing ability in tumors. The persistent luminescence hydrogel can be easily injected in vivo as a powerful localized light source for superior persistent luminescence-sensitized photodynamic therapy of tumors. The “turning solid into gel” strategy enables taking full advantages of persistent luminescence for biological applications, and shows great potential in utilizing diverse theranostic agents regardless of hydrophilicity and hydrophobicity.
\end{abstract}

1. Introduction

Photodynamic therapy (PDT) with specific spatiotemporal selectivity and minimal invasiveness has become an emerging solution for cancer therapy from fundamental research to clinic [1,2]. Revolutionary technologies that shift the excitation sources of PDT from ultraviolet/visible (UV/vis) light to near-infrared (NIR) light [2], upconversion fluorescence [3,4], NIR two-photon excitation [5,6], X-ray radiation [7], self-illumination (chemiluminescence [8,9], bioluminescence [9,10], and Cerenkov luminescence [11]) greatly promote the flourishing development of PDT with superior therapeutic efficacy [2]. However, these technologies suffer from the risks of ionizing radiation injury, tissue ablation induced by long-term irradiation and insufficient self-illumination efficiency [1,2]. Persistent luminescence (PersLum) material is a kind of optical material that can emit luminescence after the ceasing of exciting light [12–17]. The optical excitation energy can be trapped at the specific defects of PersLum material during excitation and slowly released as afterglow after excitation. The unique NIR persistent luminescence and the renewability of PersLum not only permit biosensing [18–24] and rechargeable bioimaging without the need of in situ excitation [25–47], but also can serve as an internal light source for repeated long-term photodynamic therapy (PDT) in deep tissues [48–54] and other theranostic applications [55–65]. Excellent PersLum properties play a crucial role in biological applications of persistent luminescence material [12–15]. Traditional PersLum-sensitized PDT required PersLum material with small size and favorable water solubility, which can be easily dispersed in water for intravenous and subcutaneous injections. However, there is a conflict between uniform morphology/small size and intense and long-lasting afterglow of PersLum material, and this troublesome problem has not been solved completely up to now [12–15]. For instance, high temperature sintering is the most efficient method for improving the PersLum performance, but results in agglomerated products with irregular morphologies (high temperature sintering PersLum material was defined as “PLM”).

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https://doi.org/10.1016/j.biomaterials.2019.119328

Received 15 March 2019; Received in revised form 29 June 2019; Accepted 30 June 2019

Available online 02 July 2019

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Subsequent corrosion under alkali condition and screening are necessary to obtain small-size and water-soluble persistent luminescence nanoparticles (PLNPs), but lead to 90% mass loss of PLM and remarkable decrease of luminescence performance due to the damage of crystal lattices [12-15]. While hydrothermal method without high temperature calcining is employed to produce with small size and good water solubility (H-PLNPs), but leads to a much lower PersLum performance [12-15]. Besides, multiple surface modifications, such as amination and PEGylation, are always necessary to improve water solubility, biocompatibility and targeting ability for PersLum-sensitized PDT [12-15]. Recently, a novel solid PLM implant has been developed for irradiation-free PDT, but the invasive transplantation of solid implants requires complicated and professional operation, brings potential side effects induced by invasive administration, and has difficulty in the treatment of deep tumors [51]. Therefore, it is highly desired to develop a smart strategy to take full advantages of PersLum for PDT, unifying the high utilization efficiency of PLM, performance PersLum, and facile synthesis and administration processes.

Herein, we show a facile and smart “turning solid into gel” strategy to fabricate PersLum material hydrogel (PLM-hydrogel) for high-efficient PersLum-sensitized PDT (Scheme 1). The homogeneous PLM-hydrogel was prepared by simply dispersing the high temperature calcined PLM without corrosion under alkali condition and screening into alginate-Ca\textsuperscript{2+} hydrogel, which is formed by mixing FDA approved alginate solution and Ca\textsuperscript{2+} [65-67]. The intense PersLum and long afterglow of PLM synthesized under high temperature was retained to the maximum degree, and the utilization efficiency of PLM reached 100%. The as-prepared PLM-hydrogel with good syringeability can be effortlessly injected in tumor sites of mice in vivo without the leakage from the path of the needle. The intelligent introduction of PLM with a big size into tumors via hydrogel injection facilitates the accumulation of PLM in tumors and minimizes the side effects induced by the penetration into bloodstream and other tissues of PLM. The PLM-hydrogel with intact PersLum of PLM and red light renewability as a robust in vivo imaging mode. Red light illuminations (0.7 W/cm\textsuperscript{2}) were used for the re-activation of solid persistent phosphors and persistent phosphor hydrogel, respectively. The absorbance for the MTT assay was recorded on an IVIS imaging system (Caliper Co.) under bioluminescence imaging mode. Red light illuminations (0.7 W/cm\textsuperscript{2}) were used for the re-activation of solid persistent phosphors and persistent phosphor hydrogel, respectively. The absorbance for the MTT assay was recorded on a microplate reader (Bio-tec, USA Park, CA).

2. Experimental section

2.1. Chemicals

All chemicals were at least of analytical grade and used without further purification. Ultrapure water was provided by Hangzhou Wahaha Group Co. Ltd (Hangzhou, China). Zinc nitrate, gallium oxide, germanium oxide, chromium nitrate, ytterbium nitrate, erbium nitrate, Na\textsubscript{2}HPO\textsubscript{4}·12H\textsubscript{2}O, NaH\textsubscript{2}PO\textsubscript{4}·2H\textsubscript{2}O, nitric acid, cetyltrimethylammoniumbromide (CTAB), ammonia, sodium alginate, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Indocyanine Green (ICG), 2’7’-Dichlorodihydrofluorescein diacetate (DCFH-DA) and 1,3-Diphenylisobenzofuran (DPBF) were bought from Aladdin Reagent Co. Ltd. (Shanghai, China). Ce6 was obtained from J&K Scientific (Beijing, China). DMSO was supplied by Concord Technology (Tianjin, China).

2.2. Characterisation

X-ray diffraction (XRD) patterns were measured on a D/max-2500 X-ray diffractometer (Rigaku, Japan). The scanning electron microscope (SEM) images of PLM and PLM-hydrogel were characterized by a Merlin Compact Field-Emission-Scanning Electron Microscope (Carl Zeiss, Germany). Rheological tests were performed at 25 °C using a DHR-2 rheometer (TA Instruments, U. S. A). The absorption spectra were obtained on a UV-3600 UV-vis-NIR spectrophotometer (Hitachi, Japan). The persistent luminescent imaging of PLM, PLNPs, H-PLNPs, PLM-hydrogel, PLM-hydrogel pattern, PLNPs-hydrogel, and H-PLNPs-hydrogel were carried out on an IVIS imaging system (Caliper Co.) under bioluminescence imaging mode. Red light illuminations (0.7 W/cm\textsuperscript{2}) were used for the re-activation of solid persistent phosphors and persistent phosphor hydrogel, respectively. The absorbance for the MTT assay was recorded on a microplate reader (Bio-tec, USA Park, CA).
2.3. Preparation of PLM, H-PLNPs and PLNPs

PLM (Zn1.25Ga1.5Ge0.25O4: 0.5%Cr3+, 2.5%Yb3+, 0.25%Er3+, ZGO: Cr3+, Yb3+, Er3+) was synthesized by a hydrothermal method in combination with sintering in air. Firstly, Ga2O3 was dissolved in nitric acid under hydrothermal conditions at 120 °C, and Ge2O3 was dissolved in dilute ammonia solution (3%). 0.375 mM of chromium nitrate (0.1 M), 12.5 mL of zinc nitrate (0.5 M), 1.875 mL of ytterbium nitrate (0.1 M), 1.875 mL of erbium nitrate (0.01 M) and 6.25 mL ammonium germanate (0.2 M) were added to 7.5 mL of gallium nitrate solution (1 M) in order according to the chemical formula of Zn1.25Ga1.5Ge0.25O4·0.5%Cr3+, 2.5%Yb3+, 0.25%Er3+ under vigorous stirring. After the addition of 80 mg cetyltrimethyl ammonium bromide (CTAB), the resulting solution was adjusted to pH = 8.0. The reaction solution was then ultrasonicated for 30 min, stirred for 1 h, and sealed into a 30 mL teflon-lined autoclave at 120 °C for 15 h. The resulting products was washed orderly with ultrapure water and ethanol, and then lyophilized to obtain H-PLNPs. The dried H-PLNPs was sintered in air at 1000 °C for 1.5 h and grinded in quartz to obtain PLM solid powder for further use.

To obtain PLNPs, the PLM powder was wet grinded in minimum ethanol, dispersed in 5 mM NaOH and vigorously stirred for 24 h, and the resulting colloid solution was centrifuged at 10000 rpm for 5 min to remove NaOH. The collected precipitation was redispersed in water under ultrasonication, and centrifugated at 3500 rpm for 5 min to remove large size particles. The PLNPs in resulting supernatant were collected by centrifugation at 10000 rpm for 5 min and washed with water, and the obtained PLNPs were freeze-dried for further study.

2.4. Preparation of PLM-hydrogel, H-PLNPs-hydrogel and PLNPs-hydrogel

PLM, PLNPs or H-PLNPs solid (8 mg) was dispersed in ALG solution (10 mg/mL, 0.9 mL) respectively and kept magnetically stirring for 2 h at room temperature. Then CaCl2 solution (0.1 mL, 10 mg/mL) was dropwise added into mixture to fabricate PLM-hydrogel, PLNPs-hydrogel and H-PLNPs-hydrogel.

2.5. The stability assessment of PLM-hydrogel in different media

PLM-hydrogel (8 mg/mL) was incubated with various media including normal saline, phosphate buffer solution (PBS), and DMEM culture medium, and the stability of PLM-hydrogel was monitored by taking photos for 25 days.

2.6. In vitro singlet oxygen (1O2) detection

The generation of 1O2 was measured by the absorption decrease of DPBF as a classic probe. The photostability of free DPBF (25 μg/mL; Ce6 (2 μg/mL)) was firstly evaluated after red irradiation for different times, and the absorption of DPBF and Ce6 mixture in ethanol were recorded as control group. Pre-excited PLM, PLNPs and H-PLNPs (15 mg) were added to 3 mL DPBF-Ce6 solution (DPBF: 25 μg/mL; Ce6: 2 μg/mL), and the supernatant was collected by centrifugation at different time points (5 and 10 min respectively). The absorbance of the supernatant was measured to evaluate PersLum sensitized generation ability of 1O2.

To further investigate the renewable 1O2 generation ability of persistent phosphors, Pre-excitation PLM, PLNPs and H-PLNPs (5 mg PLM/mL) suspended in water were added to the DPBF-Ce6 ethanol solution. The supernatant and persistent phosphor precipitate were collected by centrifugation respectively at 10 min. The absorbance of the supernatant was recorded to evaluate the generation of 1O2, and persistent phosphor precipitate was irradiated with red light (0.7 W/cm², 5 min) to re-activate their PersLum. The above-mentioned corresponding supernatant was then mixed with re-activated PLM, PLNPs and H-PLNPs for 5 min, and the new supernatant was collected for the measurement of its absorbance to evaluate the renewable 1O2 generation ability of different persistent phosphors.

2.7. In vitro toxicity assessment of PLM-hydrogel and Ce6

The cytotoxicities of PLM-hydrogel and Ce6 were investigated via a standard MTT assay using 4T1 cells. 4T1 cells were seeded in 96-well plates at a density of 1×10⁴/well and cultured in DMEM supplemented with 10% FBS at 37 °C in 5% CO2 for 24 h. Then the wells were washed with PBS and incubated with fresh media containing different concentrations of PLM-hydrogel (2, 4, 6, 8 and 10 mg PLM/mL) or Ce6 (0.05, 0.1, 0.2, 0.4 and 0.8 mg/mL). After cultured for 24 h, state media were replaced by fresh culture media containing MTT (10 μL, 5 mg/mL) and incubated at 37 °C for another 4 h. Then the old media were discarded, followed by the addition of 120 μL DMSO to dissolve the formed formazan crystals. The absorbance of each well was determined by a microplate reader at the wavelength of 490 nm. The cell viability was calculated using the following formula: Cell viability (%) = (Mean of absorption of treatment group/mean absorption of control group) × 100%.

2.8. Singlet oxygen (1O2) generation at cellular level

4T1 cells were seeded in 96-well plates at a density of 1×10⁴/well and cultured in DMEM supplemented with 10% FBS at 37 °C in 5% CO2 for 24 h. The cells were then exposed to different treatments: PBS (control group); pre-excitation PLM-hydrogel; Ce6 for 6 h; Ce6 for 6 h + pre-excitation PLM-hydrogel; Ce6 for 6 h + red light irradiation (0.7 W/cm², 3 min); Ce6 for 6 h + red light irradiation (0.7 W/cm², 3 min); and Ce6 for 6 h + pre-excitation PLM-hydrogel + red light irradiation (0.7 W/cm², 1 min three times with an interval of 1 h). The concentrations of Ce6 and PLM-hydrogel used in each group were fixed to 0.2 mg/mL and 0.8 mg PLM/mL respectively. The state media were replaced by the fresh culture media containing DCFH-DA (1 μM) for 20 min, and then cellular fluorescent imaging was carried out to evaluate 1O2 generation via an inverted fluorescence microscope.

2.9. In vitro PDT evaluation

The PersLum sensitized repeated PDT effect in vitro of PLM-hydrogel was assessed via a MITT assay using 4T1 cells. 4T1 cells were seeded in 96-well plates at a density of 1×10⁴/well and cultured in DMEM supplemented with 10% FBS at 37 °C in 5% CO2 for 24 h. The cells were then exposed to different treatments: PBS (control group); pre-excitation PLM-hydrogel; Ce6 for 6 h; Ce6 for 6 h + pre-excitation PLM-hydrogel; Ce6 for 6 h + red light irradiation (0.7 W/cm², 3 min); Ce6 for 6 h + red light irradiation (0.7 W/cm², 3 min); and Ce6 for 6 h + pre-excitation PLM-hydrogel + red light irradiation (0.7 W/cm², 1 min three times with an interval of 1 h). The concentrations of Ce6 and PLM-hydrogel used in each group were fixed to 0.2 μg/mL and 0.8 μg PLM/mL respectively. After the incubation for 18 h, the state media were replaced by the fresh culture media containing 5 mg/mL MTT and cultured at 37 °C for 4 h. Thereafter, 120 μL DMSO per well was added to dissolve the formazan crystals, and the absorbance at 490 nm of each well was recorded by a microplate reader. The cell viability was determined to evaluate PersLum sensitized repeated PDT effect of PLM-hydrogel.

2.10. Animal models

All small animal experiments were approved by the Tianjin Medical University Animal Care and Use Committee. The tumor models were established by the subcutaneous injection of 4T1 cells into the backs of the Balb/c mice (Beijing HFK Bioscience Co. Ltd., China). In vivo imaging and therapy were performed when the tumor size reached to 0.5–0.8 cm³.
2.11. In vivo PersLum imaging observation of PLM-hydrogel

The mice bearing 4T1 tumors were intratumorally injected with 200 μL pre-formed PLM-hydrogel (8 mg PLM/mL) with a syringe, which was pre-excited by a 254 UV lamp for 5 min. The PersLum imaging before and after injection was performed on an IVIS imaging system at different time points (5, 30 and 60 min). Red light illumination (1 W/cm²) was conducted on the tumors of mice for 10 min before re-activated PersLum imaging at 1 h, 1 d, 3 d, 5 d and 7 d after PLM-hydrogel injection.

2.12. In vivo PersLum sensitized PDT of tumors using PLM-hydrogel

To investigate in vivo PDT, the mice bearing 4T1 tumors were randomly divided into five groups (n = 3 in each group) for different treatments as follows:

Group A: Control group without the administration of any agents and light illumination;
Group B: Intravenous injection of Ce6 (200 μg/mL);
Group C: Intravenous injection of Ce6 (200 μg/mL) and red light illumination (1 W/cm², 10 min) after 2 h;
Group D: Intravenous injection of Ce6 (200 μg/mL) and intratumoral administration of 254 UV lamp pre-excitation PLM-hydrogel (200 μL, 8 mg PLM/mL) with for 5 min after 2 h;
Group E: Intravenous injection of Ce6 (200 μg/mL), intratumoral administration of 254 UV lamp pre-exicted PLM-hydrogel (200 μL, 8 mg PLM/mL) with for 5 min after 2 h, and red light illumination (1 W/cm², 10 min) after 3 h.

The images of mice were taken by digital camera to monitor the tumor change. The tumors volumes of mice with various treatments were measured by a caliper according to the following formula: tumor volume (V) = (length) × (width)²/2. Relative tumor volume was calculated as V/Vo, where V means tumor volume after treatment and Vo refers to tumor volume before treatment.

During the tumor monitoring, the body weights of mice in each group were also recorded. Besides, Kuming mice were treated with or without intravenous injection of Ce6 (200 μg/mL) and subcutaneously injection of PLM-hydrogel (200 μL, 8 mg PLM/mL), the major organs (heart, liver, spleen, lung and kidney) of mice in the control and treatment groups were collected and fixed by 4% paraformaldehyde at 1, 7, 15 day. The histopathological study was performed by the hematoxylin–eosin (H&E) staining and analyzed by optical microscope.

3. Results and discussion

3.1. Synthesis and characterization of PLM, PLNPs and H-PLNPs

PLM with bright PersLum, ZGGO: Cr³⁺, Yb³⁺, Er⁵⁺, was synthesized by a classic hydrothermal process in combination with high temperature calcining [35]. The synthesized PLM exhibited a spinel cubic phase structure (JCPDS 25–1240) without other phases of impurities such as ZnO, GeO₂, and Ga₂O₃ by X-ray diffraction (XRD) pattern analysis (Fig. 1a) [35]. Scanning electron microscope (SEM) images showed the PLM had an agglomerated irregular morphology due to the high temperature sintering (Figs. S1a and S1b). The excitation spectrum of PLM gave four bands derived from the characteristic peaks of Er³⁺, Cr³⁺ and Yb³⁺ in a wide range of spectral regions from 200 nm to 600 nm (Fig. 1b) [35]. The excitation band at 254 nm was attributed to the combination of the ZGGO host excitation band and the O-Cr charge transfer band [35]. The other three bands at 410 nm, 463 nm, and 570 nm resulted from the 3d intrashell transitions of Cr³⁺, i.e. ³A₂→⁴T₁ (τe²), ³A₂→⁴T₁ (τe²), and ³A₂→⁴T₂ transition, respectively [35]. The PLM gave a NIR emission band ranging from 600 to 800 nm with an emission peak at 695 nm under the excitation at 254 nm (Fig. 1b) [35]. The PLM had a brighter NIR fluorescence compared to PLNPs and H-PLNPs, and the much stronger PersLum of PLM can be clearly observed by naked eye (Fig. 1c). Quantitative analysis further revealed the PersLum intensity of PLM was at least 2 and 300 times higher than PLNPs and H-PLNPs respectively after 7 min past since excitation stopped (Fig. 1d). In addition, PersLum images revealed all of these three materials can be reactivated by mild red light illumination repeatedly (Fig. 1e), and recharged PLM still exhibited much stronger PersLum than PLNPs and H-PLNPs (Fig. 1e). The superior PersLum makes PLM an ideal light source for PDT application instead of PLNPs and H-PLNPs.

3.2. Synthesis and characterization of PLM-hydrogel, PLNPs-hydrogel and H-PLNPs-hydrogel

Despite appealing afterglow of PLM, the agglomerated irregular morphology makes it impossible for biological applications. Thus current PersLum sensitized PDT was limited to the usage of PLNPs and H-PLNPs with much weaker PersLum. The introduction of hydrogel to transform PLM from solid state to injectable hydrogel provides a promising way for taking full advantages of PersLum. Sodium alginate hydrogel (ALG) with excellent biocompatibility, which is formed by simply mixing Ca²⁺ and FDA approved alginate in aqueous solution, is extensively used in various biological applications, such as drug delivery, cell transplantation, and biomedical engineering. The PLM-hydrogel with good stability was fabricated by simply dispersing PLM in ALG hydrogel (Scheme 1 and Fig. S2), and the syringeability, hardness and viscosity of PLM-hydrogel can be readily tuned by changing the amounts of Ca²⁺ and PLM. Compared to the 90% mass loss in corrosion and screening processes in the synthesis of widely used PLNPs, the PLM-hydrogel with 100% of utilization efficiency of PLM showed great superiority in reducing time, cost, and energy consuming. Higher hardness and viscosity favored the PLM loading efficiency, but was adverse to syringeability of the PLM-hydrogel. To compromise high hardness/viscosity and syringeability, the optimal amounts of Ca²⁺ (1 mg/mL) and PLM (8 mg/mL) was used for the following studies (Fig. 2a). The rheology of PLM-hydrogel showed that G’ is higher than G″, which revealed the proposed PLM-hydrogel is indeed a gel (Fig. S3) [68]. SEM images indicated the aggregate PLM became uniform nanoparticles with an average size of approximately 200 nm, and were homogeneously dispersed in the PLM-hydrogel (Fig. 2b and Figs. S1c and S1d). This morphology transformation was attributed to the strong coordination interaction between PLM and ALG, greatly facilitating the homogeneous distribution of PLM in PLM-hydrogel for effective PDT. The stability of PLM-hydrogel incubated with various media including normal saline, PBS, and DMEM was evaluated for 25 days (Fig. S4). The photos revealed the hydrogel only gradually swelled along with the time and enabled maintaining their homogeneous structure for a long time due to its good stability.

Moreover, the good syringeability of as-prepared PLM-hydrogel enabled the formation of PLM-hydrogel pattern (TMU) easily, and the fluorescent and PersLum images of PLM-hydrogel both proved their attracting afterglow property, favorable syringeability and homogeneity (Fig. 2c–f). Furthermore, various volumes (20–100 μL) of PLM-hydrogel with excellent reproducibility and homogenous luminescence can be readily extruded (Fig. 2g and h), facilitating the administration in biological application in vivo.

3.3. Optical properties of PLM-hydrogel

PLM-hydrogel can inherit the PersLum of PLM to the maximum extent due to the retention of intact crystal structure during the mild mixing process without the need for corrosion and screening. The much stronger fluorescence of PLM-hydrogel can be observed under UV lamp compared to PLNPs-hydrogel and H-PLNPs-hydrogel (Fig. 2i–n). The excitation and emission spectra of PLM-hydrogel were similar to PLM owing to the minimized influence on the luminescent features of PLM.
during the formation of PLM-hydrogel (Fig. 3a). The synthesized PLM-hydrogel exhibited bright PersLum in NIR region, and the PersLum intensity of PLM-hydrogel was at least 1.7 times higher than PLNPs-hydrogel and 200 times higher than H-PLNPs-hydrogel after 25 s past since excitation stopped (Fig. 3b). The long-lasting afterglow of PLM-hydrogel can be detected even after 24 h in PersLum imaging (Fig. 3c). The PersLum of PLM-hydrogel can be repeatedly reactivated by mild red light illumination (Fig. 3c and d), and the reactivated PersLum of PLM-hydrogel was still much brighter than PLNPs-hydrogel and H-PLNPs-hydrogel (Fig. 3c). These results clearly testified the appealing syringeability and superior PersLum of PLM-hydrogel, showing great potential in PersLum sensitized PDT.

3.4. In vitro generation of 1O2

Photosensitizers play a crucial role in PDT, which can convert O2 to cytotoxic singlet oxygen (1O2) under light irradiation. In our study, chlorin e6 (Ce6) with high PDT efficiency was chosen as a model photosensitizer. The overlap of absorption of Ce6 as a model photosensitizer and luminescence of PLM permits NIR PersLum sensitized PDT without the need for continuous light irradiation (Fig. 4a). 1,3-Diphenylisobenzofuran (DPBF) as a classic probe was used for the determination of the generation of 1O2, which can destroy the structure of DPBF and lead to a decrease of its absorption at 410 nm [69]. Free Ce6 did not give rise to the absorption decrease of DPBF unless red light irradiation was applied (Figs. S5–7). In contrast, pre-excitation PLM led to remarkable decrease of absorption of DPBF in the presence of Ce6 without the need for red light illumination, indicating the effective generation of 1O2 triggered by PersLum instead of continuous light irradiation (Fig. 4b and Fig. S8). In addition, PLM-induced production of 1O2 was renewable after mild red light illumination, guaranteeing repeated PDT capability to realize high therapeutic efficacy. The generation ability of 1O2 induced by PersLum highly depended on the PersLum intensity of PLM, and PLNPs and H-PLNPs with much weaker PersLum only triggered the production of less 1O2 (Fig. 4b, Figs. S9 and S10).

3.5. Cytotoxicity and PersLum sensitized PDT in vitro

The cytotoxicity of PLM and PLM-hydrogel was evaluated by a standard MTT assay using 4T1 cells. Over 90% cancer cells remained alive even after the incubation with 0.8 mg/mL of PLM and PLM-hydrogel for 24 h, demonstrated their excellent biocompatibility (Fig. 4c). PersLum-sensitized generation of 1O2 in cellular level was then evaluated by cellular fluorescent imaging using a DCFH-DA probe (Fig. S12). The results revealed pre-excited PLH or red light both can lead to a remarkable generation of 1O2 in Ce6-treated cancer cells, and the combination treatment of pre-excited PLH and red light is capable of inducing the maximum amounts of 1O2 in Ce6-treated cancer cells. The PLM with good biocompatibility and high generation capability of 1O2 was then employed for PDT in vitro. 4T1 cells were exposed to control, Ce6, Ce6 + red light, Ce6 + pre-excitation PLM-hydrogel, and Ce6 + pre-excitation PLM-hydrogel + red light, and the cell viabilities were determined by a MTT assay (Fig. 4d and Fig. S11). Fig. 4d revealed only pre-excitation PLM-hydrogel or Ce6 can’t lead to obvious cell death, and 53% of cancer cells were damaged after the treatments of Ce6 and red light based on traditional PDT. In contrast, the pre-excitation PLM-hydrogel plus Ce6 led to 40% of cell death without the need for external light source, and further introduction of red light re-activation (0.7 W/cm2, 3 min) remarkably improved the therapeutic efficacy and 82% of cells were destroyed due to the renewability of PersLum sensitized PDT. Moreover, giving an 1-min red light illumination every hour for 3 times also resulted in a similar cell-killing effect via PersLum-sensitized PDT, which is an alternative recharging...
approach to 3-min red light re-activation, and the flexible choice of repeated PDT strategy facilitates the potential clinical applications. The above results prove the low cytotoxicity of PLM and PLM-hydrogel, and effective PersLum-sensitized PDT.

3.6. PersLum imaging, PersLum-sensitized PDT and toxicity evaluation in vivo

We then investigated the feasibility of PersLum sensitized PDT in vivo using PLM-hydrogel. High accumulation of theranostic agents in tumors and low leakage to surrounding tissues play a crucial role in high-efficient tumor theranostics with minimal side effects. The smart design of turning solid PLM into injectable PLM-hydrogel not only allowed the injection without leakage of PLM from the path of needle, but also permitted the introduction of PLM with big size into tumors (Fig. S13). In contrast, the injected ICG solution leaked out from path of needle obviously. The strong fixing ability in tumors of PLM with big size greatly benefited the high PDT efficacy, and minimized the
potential safety threats induced by the penetration into bloodstream and other tissues. For in vivo application, the pre-excited PLM-hydrogel was injected to the tumors of the mice, and the strong PersLum of PLM-hydrogel can be observed from optical imaging clearly (Fig. 5a). The PersLum can be readily reactivated by red light illumination at 1 h, and even 7 days after the administration of PLM-hydrogel. The pre-excited PLH showed intense initial and re-activated PerLum both with high signal-to-noise ratios indicated the excellent PersLum feature and outstanding renewability (Fig. 5a). Moreover, the long-lasting bright PerLum was mainly present in the tumor region for a long time, demonstrating the outstanding fixing ability in tumors of PLM that can serve as a powerful inert light source for PDT (Fig. 5a). The mice bearing tumors were intravenously injected with Ce6, and fluorescent imaging indicated the Ce6 was mainly accumulated in tumors at 2 h (Fig. S14). The intratumoral injection of pre-excitation PLM-hydrogel and red light irradiation were then employed to trigger PDT at 2 h after the administration of Ce6 (Fig. 5b and c and Fig. S15). PLM-stimulated PDT exhibited a similar tumor suppression effect as external light source, revealing that PLM can serve as an excellent internal light for PDT instead of continuous light irradiation. Furthermore, the mild red
right irradiation (1 W/cm², 5 min) enables the renewability of PLM and further improves the tumor therapeutic efficacy remarkably. In contrast, the tumors of mice in the control group kept growing quickly all the time. The sizes of tumors in mice treated with PLM-PDT with red light re-activation were 16 times smaller than those in the control group, proving the appealing PDT efficacy of PLM (Fig. 5c).

To evaluate in vivo toxicity, body weight change monitoring and histopathological analysis of major organs were performed after various treatments. The body weight change of mice after PDT did not exhibit obvious difference from the control group, revealed the low toxicity in vivo of PLM and PLM-hydrogel (Fig. 5d). Hematoxylin and eosin (H&E) staining results indicated the major organs of mice (including heart, liver, spleen, lung, and kidney) did not show obvious histopathological change compared to the normal mice, confirming the excellent biocompatibility of PLM and PLM-hydrogel and biosafety of PLM-hydrogel triggered PDT (Fig. S16).

Fig. 5. In vivo PersLum imaging and PLM-hydrogel triggered PDT. (a) Persistent and renewable luminescent imaging in vivo. (b) The photos of tumors in mice with different treatments (group A: Control; group B: Ce6; group C: Ce6 + red light; group D: Ce6 + PLM; group E: Ce6 + PLM + red light). (c) The relative volumes of tumors in mice with different treatments (**p < 0.01, ***p < 0.001). (d) The body weight monitoring of mice with different treatments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
4. Conclusion

In summary, we developed a facile and smart “turning solid into hydrogel” strategy to synthesize PLM-hydrogel to take full advantages of PersLum for high-efficient PDT. The homogeneous PLM-hydrogel was synthesized by simply dispersing high temperature sintering PLM into ALG-Ca\(^{2+}\) hydrogel. Compared to traditional PLNPs and H-PLNPs with 90% loss of mass and weak PersLum, the PLM-hydrogel not only had 100% of utilization efficiency of PLM, but also maintained the intact PersLum of PLM. The proposed PLM-hydrogel possessed good syringability, appealing biocompatibility, intense NIR PersLum, and red light renewability, which can trigger the continuous and renewable production of \( \text{O}_2 \). The PLM-hydrogel can be noninvasively injected into body without the leakage from the path of needle, and the introduction of PLM with a big size exhibited strong fixing ability in tumors, favoring therapeutic efficacy and bioassay. Finally, the PLM-hydrogel with bright PL and red light renewability was applied in PersLum-sensitized repeated PDT in vitro and in vivo successfully. We believe the proposed strategy of turning solid to hydrogel not only takes full advantage of PersLum for superior PDT, but also lays down a novel way to make theranostic agents become bioavailable regardless of their hydrophilic and hydrophobic features.

5. Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

6. Competing financial interests

The authors declare no competing financial interests.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grants 81671676, 21435001), National Science Foundation of Tianjin City (No. 18JCYBJC20800).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biomaterials.2019.119328.

References


