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Thermosensitive nanocomposite gel for intra-tumoral two-photon photodynamic therapy

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Abstract

We propose here a new approach to achieve intratumoral near-infrared (NIR) two-photon photodynamic therapy (PDT). We established a composite micellar thermosensitive hydrogel made of clinically approved methoxy poly(ethylene glycol)-polylactide copolymer (mPEG-PDLLA) and Pluronic (F127). The mPEG-PDLLA form micelles that can be loaded with two-photon absorption compound (T1) and photosensitizer (PS). The F127 micelles are liquid at room temperature and while forming an hydrogel at body temperature. This enables an in situ gelification upon injection providing long-term retention within the tumor. The NIR light is thus upconverted into visible light by T1 and excited PS. The morphology, rheology properties and releasing profiles of hydrogel were fully characterized. The rheology properties and releasing mechanism was investigated. The composite hydrogel showed significant cytotoxicity to 4T1 murine breast cancer cells upon NIR laser irradiation, while it showed non-significant cytotoxicity without. Time-dependent in vivo and ex vivo distribution results suggested that hydrogel administrated via intra-tumoral injection could prolong both PDT agents retention in tumor. We show here that the use of NIR radiation allows deep tissue penetration and inhibition of tumor growth of more than 50% even under 1 cm thick muscle tissue.

Keywords: Photodynamic therapy; Two-photon absorption; hydrogel; micelles; deep penetration.

Introduction

Photodynamic therapy (PDT) is based on the administration of a drug that sensitizes cells to specific wavelength of light. Photosensitizer (PS), and the light radiation are harmless on their own but when combined, several reactive oxygen species (ROS) are formed triggering (i) cell apoptosis, (ii) localized ischaemia and (iii) acute local inflammation. The three effects make PDT a very effective anti-cancer treatment as cells can be killed locally either directly or by removing their blood supply as well as generating tumor immune memory.¹ PDT is an approved minimally invasive therapeutic procedure for non-melanoma skin, head and neck, esophageal and non-small cell lung cancer. However its expansion to other tumors is limited by the inability of light to penetrate tissues.²⁻⁴ Breast cancer, frequently happens and recurs in the skin and soft tissues of the chest wall, has large tumors, numerous small nodules or infiltrating sheets of cells in the lesions.^{5,6} In clinical trials, PDT has been used to treat recurrent and metastasis breast cancers with shallow depth. This is due to the excitation wavelength of PDT normally below 700 nm which has limited light penetration depth ranges from few millimeters up to one centimeter.⁷ Hence any improvement on either the photosensitizer efficiency or light penetration could potentially lead to major advancements in the application of PDT.⁸⁻¹¹ Most PSs, such as porphyrin, phthalocyanine and phenothiazine families, are aromatic hydrophobic compounds that aggregate in aqueous solutions with consequent reduction in ROS.¹²⁻¹⁴ PS also lack of any targeting capability leading to a systemic distribution with consequent side-effects. For example, the patient must avoid exposed under sunlight to reduce phototoxic side effects after therapy for a few days to weeks.¹⁵⁻¹⁸ One way around this is to encapsulate the PSs into carrier that can either selectively target cancer cells or

allows their internalization and localization into tumors.¹⁹ A way to improve light penetration into deeper tissues is to use two-photon near-infrared (NIR) excitation. Two-photon excitation relies on two photons, hence about half the energy than one photon, to excite a molecule that emits a higher energy fluorescence photon. In order to apply two-photon excitation to PDT, we combine a NIR two-photon probe with a PS molecules using fluorescence resonance energy transfer (FRET).²⁰⁻²² We thus apply a highly tissue-penetrant NIR two-photon radiation to excite two-photon compound whose emission transfer to a PS receptor. The delivering tools for two-photon PDT agents were generally focused on polymeric or metallic nano-vesicles and most of the researches were stopover in vitro study. Frechet reported a dendritic nanoparticle which was composed with a porphyrin core covalently linked with TPA corona and showed singlet oxygen generating in solution under 780 nm laser irradiation.²³ QingHua Xu reported that photosensitizer doped conjugated polymer nanoparticles preformed two-photon photodynamic therapy in living cells.²⁴ One of our previous study was applied pH sensitive polymeric carrier for in vivo anti-cancer attempting and such DDS was administrated via i.v..

To avoid complicate and difficult-to-regulate chemical modifications, we propose the co-encapsulation of the two molecules into a micelles formed by clinically-approved methoxy poly(ethylene glycol)-polylactide copolymer (mPEG-PDLLA). The micelle diameter is about 25nm and ensures the two probes are in close proximity to allow for FRET and consequent PDT. Recently, we reported the design, synthesis, and full optical characterization of a novel imidazole derivative, T1, which exhibit large TPA cross-section (~100 GM) in the NIR region (800-900 nm).²⁵ We combine here T1 with pyropheophorbide a (PPa), a hydrophobic PS from chlorophyll a derivatives. Pheophorbide compounds are considered a second-generation PS as they have improved properties over porphyrin based ones.²⁶ mPEG-PDLLA micelles are expected to improve the biodistribution of drug molecules and promote PDT effect by preventing PS molecules from aggregating.^{27, 28} mPEG-PDLLA micelles are biodegradable and showed good biocompatibility being based on PEG and PDLLA polymers both being classified as generally

recognised as safe by the US food and drug administration (FDA).^{29,30} Micelles can penetrate tumor very effectively and this makes them ideal when combined with targeting moieties.³¹ However, such an ability also means that they dilute and end up into other organs leading to either side-effect or diluting the PDT effect. We thus propose the design of a nanocomposite hydrogel to prolong micelle residency in the site of injection to focus PDT locally. In clinical practice, intratumoral injection has not been established as a routine administration route due to relatively rapid clearance of administrated drugs from tumors and liquid formulations are forced out by intratumoral pressure. Such composites undergo from liquid to gel within the tumor retaining the therapeutic cargo locally which avoids the initial burst release of drug from nanocarriers reducing thus systemic toxicity.³²⁻³⁴ Thermosensitive *in situ* hydrogel has the advantages of simple preparation, convenient administration and controlled drug releasing.^{35,36} In this study, T1 and PPa co-loaded mPEG-PDLLA micelles were then mixed into poly(ethylene oxide-b-propylene oxide-b-ethylene oxide) (PEO-PPO-PEO) (also Pluronic-F127). F127 (BASF), also known as Poloxamer (ICI), is a commercially available pharmaceutical excipient approved for several clinical uses.³⁵ F127 assembles into dispersed micelles and these in turn aggregates into micellar gels at temperature higher than 37°C. The consequent sol-gel transition occurs spontaneously at body temperature (i.e. 37°C) and allow for a fluid that can flow freely below room temperature and turns into a transparent gel once in contact with the body.³⁶ We described the design, preparation and characterization of the thermosensitive hydrogel/micelle composite DLM-gel for TP-PDT agents delivering against 4T1 murine breast cancer (**Scheme 1**).

Scheme 1. Schematic illustration of micelles thermosensitive hydrogel composite based two-photon photodynamic therapy against cancer

Size and morphology of T1 and PPa co-encapsulated micelles (DLM), rheology, releasing profiles and singlet oxygen generation of DLM and F127 composite gel (DLM-gel) was characterized. Cytotoxicity, live cell imaging, *in situ* ROS generation and cell morphology

collapsing were investigated. Time-dependent *in vivo/ex vivo* organ distribution and anti-cancer efficacy of DLM-gel based TP-PDT were studied on 4T1 xenograft mice. The micelle-thermosensitive hydrogel composite prevented PSs from aggregating with convenient administration, long-term tumor accumulating and prolonged therapeutic depth of TP-PDT, thus offer a PDT combination kit as potential candidate against breast cancer.

Materials and methods

Materials: Pluronic (F127, Mw= 9840~14600) was donated by BASF Co., Ltd. mPEG₄₈-PDLA₄₀ (MW 2000-2000 Da) was purchased from Jinan Daigang Biomaterial Co., Ltd. Pyropheophorbide a (PPa, Mw=534.44) was purchased from Shanghai Xianhui Pharmaceutical Technology Co., Ltd. Anthracene-9,10-dipropionic acid disodium salt (ADPA) was purchased from Santa Cruz Biotechnology. 2',7'-dichlorofluorescein diacetate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Methanol, chloroform and dimethyl sulfoxide were purchased from Sigma-Aldrich.

Cell culture and animals. All the cell lines involved in this paper were supplied by KeyGEN Ltd. The murine breast cancer cells (4T1) and Human embryonic lung fibroblasts (HELFL) cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 g/mL streptomycin , in a humidified incubator (5% CO₂/95% air atmosphere at 37 °C). Female BALB/c mice (20 ± 2 g, 6-8 weeks old) were selected for tumor model. The animals were purchased from the experimental animal centre, Chongqing University of Medicine in China, and housed in a controlled temperature room with regular alternating cycles of light and darkness. All animals were acclimatized for a week prior to the experiments and were performed in line with national regulations and approved by the animal experiments ethical committee of School of Pharmaceutical Sciences & School of Chinese Medicine, Southwest University.

Preparation of PEG-PDLLA micelles. Blank micelles (BM), PPa encapsulated micelles (PSM), T1 encapsulated micelles (TPM), T1 and PPa co-encapsulated micelles (DLM) were prepared in the similar manner via a thin-film hydration method.³⁷ To prepare DLM, T1, PPa and mPEG₄₈-PDLLA₄₀ (1:1:10, w/w/w) were dissolved in a mixture solvent of chloroform and methanol (2:1) thoroughly and then dried under rotatory evaporator at 40 °C until the film was formed. 0.01M phosphate buffer saline (PBS, pH7.4) was added dropwise to hydrate the thin-film. The solution was sterile filtered through a 0.22 µm membrane filter to remove unloaded drugs and polymer debris after stirring for 12 hours. Micelles were stored at 4 °C for further experiments, and fresh samples were prepared for each experiment.

The absorption spectra of BM, TPM, PSM and DLM were determined by a UV-6100 spectrometer (Shanghai Mapada Instruments Co., Ltd, China). The calibration curve for T₁ was $A = 0.05635 C + 0.00719$ ($R^2=0.998$), the linear range is good within the concentration range of 5.425~12.23 g/mL (Figure S1a); the calibration curve for PPa was $A = 0.05094 C + 0.03567$ ($R^2=0.996$), the linear range is good within the concentration range of 5.254~14.83 g/mL (Figure S1b). The encapsulation efficacy, E_E and drug loading, D_L were calculated as the following equations:

$$E_E = \frac{w_L}{w_0} \text{ and } D_L = \frac{w_L}{w_0 + w_M} \quad (1)$$

where w_L is the weight of the loaded drug, w_0 the initial drug weight and w_M the weight of the micelle polymer.

Micelle size, zeta-potential and morphology characterization. The size and zeta potentials of the micelles were measured with a dynamic light scattering (DLS) Zeta-sizer (Malvern Instruments, Malvern, UK) at a wavelength of 633 nm at 25 °C. Each sample was measured 13 runs per measurement and 15 seconds per run. Distinct fractions of the runs were removed from the final measurement calculation using a dust filtration algorithm. The intensity-weighted harmonic mean particle diameter (Z-average) and the polydispersity index (PI) according to ISO 22412 were used to compare sizes and size distributions of the different samples. Each measurement was performed

in triplicate. Transmission electron microscopy (TEM) images were obtained on a transmission electron microscope (JEM1200-EX, JEOL, Japan) operating at 120 kV. Samples were prepared by placing a drop of diluted CAM dispersion or AHU-1 solution on a carbon-coated copper grid (150 mesh) and drying for 10 min in air. Hydrogel structure was analyzed by scanning electronic microscopy (SEM), and the sample was lyophilized and observed with scanning electronic microscopy (S-520, Hitachi, Japan).

Two-photon cross section. Two-photon induced fluorescence method was used to determine the two-photon absorption cross-sectional area (δ). It was determined by using femtosecond laser pulse and a Ti: Sapphire system (710–930 nm, 80 MHz, 140 fs, Chameleon II). The effective δ_I of the target compound was calculated based on fluorescence quantum yield (fluorescein as the standard material, δ_0).

The calculation formula of two-photon absorption cross-section is:

$$\delta_1 = \delta_0 \frac{F_1 \phi_0 c_0}{F_0 \phi_1 c_1} \quad (2)$$

where F_1 and F_0 are the two-photon induced fluorescence integral of the sample index 1 and standard respectively index 0; ϕ_1 and ϕ_0 fluorescence quantum yield; c the sample concentration.

Determination of singlet oxygen ($^1\text{O}_2$). The singlet oxygen ($^1\text{O}_2$) generated in the cuvette was determined by using anthracene-9,10-dipropionic acid disodium salt (ADPA) as the indicator. The decrease of ADPA absorption at 400 nm reflects the generation of $^1\text{O}_2$. Sample solutions were mixed with 20 μM ADPA solution. Then the mixed solution was placed in a micro quartz cuvette under a laser beam of 808nm with an intensity of 0.5 W/cm^2 (A semiconductor solid-state laser source, manufactured from Xian Midriver Optoelectronics Technology Co., Ltd, China) excitation wavelength. The cuvette was placed in an ice water bath during irradiation. The experiment was done in triplicate and the mean values $\pm$ SD were calculated.

Preparation of DLM-gel. DLM-gel was simply made by dissolving required amounts of F127 into DLM solution (mPEG-PDLLA concentration around 10 mg/mL) at 4 °C with using the ‘cold method’.³⁸ PSM-gel and TPM-gel were prepared in the same manner by mixing PSM and TPM into F127, individually. The viscosity of gel samples was measured using a Brookfield viscometer (DV-II+Pro, MA, USA). Spindle type 62 was used at fixed shear rates 60 rpm at 4 °C. Measurements were carried out where 25 mL sample was placed in a glass tube and the rotation speed was kept constant for at least 60 s. The experiment was done in triplicate and the mean values $\pm$ SD were calculated.

Determination of gelation temperature and time. Gelation temperature $T_{\text{sol-gel}}$ of different concentration formulations were measured using a vial titling method.³⁹ A glass vial containing F127 sol was placed in a cold water bath fitted with water circulating pump for even temperature increasing. A thermometer with an accuracy of 0.1 °C was immersed in the glass vial. The measured $T_{\text{sol-gel}}$ was the mean of triplicates. The gelation time was determined according to the method of Paola et al.⁴⁰ Briefly, a 20 mL vial containing a magnetic bar and 5 mL of each formulation was immersed in a thermostated bath at 37 °C, with constant stirring at 20 rpm. The time at which the magnetic bar stopped moving due to gelation was recorded as the gelation time. The results presented are the mean of 3 separate measurements.

Rheology. Based on the linear viscoelastic regime obtained from preliminary strain/stress sweep measurements, strain value 0.05% was chosen for rest rheological experiments to ensure elastic modulus G' value constant and oscillating stress was linear with strain. The rheology of various hydrogels was measured using a rheometer (DHR-1, TA, USA). The strain scanning mode under the amplitude sweep mode was setup in a rheometer equipped with a parallel plate in the oscillation mode. Temperature sweeps (20–40 °C) were performed at a fixed frequency of 1 Hz and a heating rate of 1 °C/min. Frequency sweeps (0.1–20 Hz) were also performed at 37 °C. All the sample thickness was 500 μm . G' and G'' represented elasticity modulus and viscosity modulus, individually.

Gel erosion and drug release. The gel erosion and drug release were determined by using a membraneless model.^{41,42} In detail, 2g of DLM-gel and equivalent amount of free T1, free PPa and DLM were individually placed at the bottom of EP tube (15 mL), and 10 mL PBS (pH 7.4) containing Tween 80 (0.5%, w/v) was added. The vials were weighed after each removal of the release medium with 100 rpm shaking at 37 °C. The hydrogel erosion-time profiles were plotted, and the erosion speed was calculated. The in-vitro release profile of T1 and PPa from DLM-gel were investigated simultaneously with the erosion profiles. The concentration of T1 and PPa in the release medium was determined by UV-vis spectrometer. All the experiments were repeated for three times.

Cell viability assays. The cellular viability was determined by MTT assays, according to the manufacturer's instructions. Cells were centrifuged and counted to ensure viability and plated in flat-bottom 96-well plates. Cells were allowed to settle down overnight for light off and light on cytotoxicity assays.

Dark (light off) cytotoxicity. 4T1 and HELF Cells were plated at a density of 8000 and 10000 cells/well in flat-bottom 96-well plates, respectively. T1 (in DMSO), PPa (in DMSO), mPEG-PDLLA, F127, DLM and DLM-gel were added onto the cells with gradient concentrations and incubated for 48 h. The final DMSO content in the culture medium was no larger than 0.05% (v/v). Cells dosing with 5 μ L PBS were applied as 100% control. MTT solution (5 mg/mL, 10 μ L) was added into each well, and the medium was removed after 4 hours. This was followed by the addition of 150 μ L of DMSO to each well. The absorbance was measured at a wavelength of 570 nm with a 690 nm background subtraction by using a UV/vis plate reader. The cell viability (%) = (OD value in the experimental group / OD value of the control group) $\times$ 100%. All the experiments were repeated for six times.

Light on cytotoxicity. Cells were incubated with DLM-gel (C_{PPa} = 5.0 μ M , C_{T1} = 5.8 μ M) for 24 h and illuminated with a laser (808nm) at different intensity (0.20, 0.36, 0.50, 0.62 and 0.75 W/cm²) for 180 s and different time (0, 30, 60, 120,180 s) with 0.5 W/cm² intensity, respectively.

The MTT assays were performed in the same manner as the light-off groups. All the experiments were repeated for six times.

Live cell images of cellular uptake. To observe the cellular uptake of samples, 4T1 cells were treated with PSM-gel ($C_{PPa}=5.0\ \mu\text{M}$), TPM-gel ($C_{T1}=5.8\ \mu\text{M}$) and DLM-gel ($C_{PPa}=5.0\ \mu\text{M}$, $C_{T1}=5.8\ \mu\text{M}$) for 24 h at 37 °C. Then the cells were washed three times with cold PBS and observed under two-photon laser confocal microscope (TP-LCM) (ZEISS 710, CarlZeiss, Oberkochen, Germany). Lambda stack scanning was performed by treating the cells with TPM-gel ($C_{T1}=5.8\ \mu\text{M}$) and DLM-gel ($C_{PPa}=5.0\ \mu\text{M}$, $C_{T1}=5.8\ \mu\text{M}$) for 2 h, and the scanning range was set on TP-LCM from 396 to 716 nm with an excitation wavelength of 808 nm. The intracellular ROS level was determined by using 2',7'-dichlorofluorescein diacetate (DCFH-DA) as the probe. Briefly, cells were dosed with DLM-gel ($C_{PPa}=5.0\ \mu\text{M}$, $C_{T1}=5.8\ \mu\text{M}$) for 24 hours. The cells were washed and incubated with 10 μM DCFH-DA at 37 °C for 30 minutes. Subsequently, cells were irradiated under the TP-LCM in scanning mode (808 nm, 0.3 W/cm², 30 scans of 10 s each). The fluorescence intensity of DCF was captured (Ex=485 nm, Em=535 nm), and the relative levels of ROS were analyzed by an image processing software ImageJ.

Animal experiments. Subcutaneous 4T1 murine breast cancer model was established for *in vivo* investigation. 4T1 cell suspension was subcutaneously injected into the back of female BALB/c mouse (5×10^5 cells per mouse). Solid tumors were allowed to form over a period of 1~2 weeks.

***In vivo* and *ex vivo* fluorescence imaging.** DLM-gel and DLM were individually intratumoral injected into subcutaneous tumor bearing mice when the tumor volume reaches around 200 mm³. DLM-gel was stored on an ice pack before injection. The sample retention was observed in *in vivo* imaging system (Bruker Corporation, Karlsruhe, Germany) by using PPa as fluorescence probe (Ex=640 nm, Em=710 nm). The *in vivo* mice imaging was monitored at the time points of 12 hours, 24 hours, 2, 4, 7 and 14 days (n=3). To capture the *ex vivo* organ images, mice (n=3) were sacrificed

at set time points (12 hours, 24 hours, 2 and 4 days) and heart, liver, spleen, lung, liver and tumor were excised. The mean PPa fluorescence intensity (Ex=640 nm, Em=710 nm) of each organ was calculated using the Analysis Workstation software.

***In vivo* anti-cancer efficacy.** Subcutaneously tumor bearing mice were developed according to the method above. 42 mice were randomly divided into 7 groups (n=6) when the tumor volume reached around 200 mm³, the administration formulas and irradiation plans were demonstrated in Table 1. Each group was administrated at the 1st, 5th and 10th day and laser irradiation was performed the day after (24 hours). The injection volume of each group was 50-100 µL, and ‘Laser’ and ‘Control’ groups were injected with 100L PBS. The irradiation time was 10 min, and the light dosage for both 808 nm and 660 nm laser was 300 J/cm². In order to reduce skin damage, 5 mins rest was taken for every 5 min light irradiation. The tumor volumes were calculated by the following formula: tumor volume V (mm³) = 1/2 length (mm) width (mm) width (mm). Tumor volume and body weight were measured every 2 days. At the 21st day, animals were euthanized and sacrificed; the tumors were excised and weighted, tumor growth inhibition (TGI) value was calculated as: TGI = (1-Mean tumor weight of treatment group)/(Mean tumor weight of control group)100%.

Table 1. Different formulations.

Groups	C _{PPa} (mg/kg)	C _{T1} (mg/kg)	Laser irradiation
DLM-gel + Laser	5.2	4.9	808 nm
PSM-gel + Laser	5.2	N/A	660 nm
DLM-gel + Laser (Blocked)	5.2	4.9	808 nm
PSM-gel + Laser (Blocked)	5.2	N/A	660 nm
DLM-gel	5.2	4.9	No
Laser	N/A	N/A	808 nm
Control	N/A	N/A	No

Histological analysis. The heart, liver, spleen, lung, kidney and tumor harvested from the 21st day of each group were fixed in 4 % paraformaldehyde solution. Samples were then paraffin-embedded, sectioned into 4 µm thick slices, stained with hematoxylin & eosin (H&E). The terminal

deoxynucleotidyl transferase (TdT)-mediated dUTP Nick-End Labelling (TUNEL) is used to detect DNA fragmentation caused by apoptosis. The apoptotic cells appeared nut-brown nucleus. To preliminary investigate biocompatibility of DLM-gel, 0.2 mL DLM-gel was injected into the back leg of mice and tissue was harvested from 1st, 3rd and 7th day for H&E staining. 0.2 mL of PBS was as the control. Specimens were examined under a digital microscope (NIKON ECLIPSE TI-SR).

Statistical analysis. All the experiments were repeated at least three times. Data were expressed as mean standard deviation (SD). Results were analyzed by two-tailed Student's t-test for two groups and one-way ANOVA for multiple groups. * $p < 0.05$ or ** $p < 0.01$ was considered statistically significant.

Results and discussion

Characterization of micelles. A film hydration method was applied to prepare T1 (TPM), PPa (PSM), double drug loaded micells (DLM) and blank micelles (BM), individually. The characteristic absorption peaks of T1 and PPa at 376 nm and 710 nm (**Figure 1a**), respectively, indicating that both hydrophobic compounds were encapsulated into mPEG-PDLLA micelles. The energy transfer between T1 and PPa is critical to the TP-PDT process. Encapsulating together of both donor (T1) and receptor (PPa) into the micelles reduced energy loss as forces the molecules within a space with a diameter lower than the required distant for efficient energy transfer. In addition, there was a substantial overlapping between the UV absorption spectrum of PPa and the fluorescence emission spectrum of T1 (**Figure 1b**). Moreover, T1 loaded micelles showed large two-photon absorption cross-section at 808 nm ($\delta = 293.7 \text{ /GM}$, inset). The conditions above could facilitate the FRET effect and thus augmented the TP-PDT efficiency.

Table 2. Characteristics of BM, PSM, TPM and DLM. Data are represented as mean $\pm$ SD ($n = 3$). ^a EE and DL are short for encapsulation efficiency and drug-loading capacity. ^b Average diameters of micelles. ^c Polydispersity index (PDI)

Formulations	EE ^{PPa} (%) a	EE ^{T1} (%) a	DL ^{PPa} (%) a	DL ^{T1} (%) a	Size (nm) b	PDI (m ² /G ²) c	Zeta-potential (mV)
BM	N/A	N/A	N/A	N/A	22.7 ± 1.3	0.192	-12.3 ± 1.8
PSM	82.6 ± 2.1	N/A	7.5 ± 1.6	N/A	27.1 ± 3.0	0.154	-10.8 ± 2.6
TPM	N/A	76.3 ± 1.5	N/A	6.3 ± 1.1	26.2 ± 2.3	0.135	-11.7 ± 3.5
DLM	73.2 ± 3.2	70.5 ± 1.9	6.8 ± 2.2	6.0 ± 1.3	31.5 ± 4.3	0.169	-12.0 ± 2.2

The encapsulation efficiency of T1 and PPa in DLM were more than 70%. The average size of DLM determined by DLS was 31.5 ± 4.3 nm with PDI of 0.169. (Table. 2) DLM showed spherical shape in TEM images with a measured diameter of 28.1 ± 4.9 nm. (Fig. 1c) The smaller size of DLM in TEM due to the micelles retained the hydration layer under the pre-treatment conditions of DLS. The zeta potential value of DLM was -12.0 ± 2.2 mV and the negatively charged surface bodes well for avoiding being recognized by macrophages in endothelial reticulum system and extend the time in the blood circulation.⁴³ TEM images and DLS figures of the micelle samples could be found in supplementary Figure S2.

Figure1. (a) UV spectra of TPM, PSM and DLM, **(b)** Normalized spectra of TPM emission and PSM absorption and TPA cross-section of TPM, **(c)** size distribution of DLM and TEM morphology was inserted.

Optimization of DLM-gel and rheological properties study. F127 micelles are dispersed under room temperature and converts to a semi-solid hydrogel in response to the rise in temperature *in vivo*. Micelles-hydrogel composite was formed by mixing F127 into DLM solution (**Figure. 2a**, inset). As observing under SEM, DLM-gel showed three-dimensional network spatial structure with consistent pore size (**Figure. 2a**). DLM micelles in composite maintained the core-shell structure with an average diameter of 48.3 ± 11.6 nm from TEM images (Fig. 2a, inset). According to Table 3, $T_{\text{sol-gel}}$ of DLM-gel decreased while the concentration of F127 increased. Meanwhile, the gelation time and viscosity of corresponding composite formulations were decreased and increased, respectively. These results are in a good agreement with the characteristic of piroxicam loaded

poloxamer hydrogels.⁴⁴ The rheology investigation of DLM-gel was validated this result. The elastic modulus G' value of DLM-gel was suddenly increased at $T_{\text{sol-gel}}$. (**Figure 2b**) The optimized hydrogel formulation was composed of 16% of F127 (w/w) in DLM solution (mPEG-PDLLA concentration 10 mg/mL) and the $T_{\text{sol-gel}}$ was 29.7 ± 0.4 °C. The optimized DLM-gel maintained a flowing liquid state below room temperature (25 °C) and was able to transform into hydrogel at body temperature (37 °C). Moreover, the viscosity of the optimal DLM-gel was 115.1 ± 6.2 mPa·s at 4°C which is convenient for injection. The viscous modulus G'' refers to the energy lost when the material is deformed due to viscous deformation, which reflects the viscosity of the material. In **Figure 2c**, the results showed that the G' value was much larger than the G'' value and the difference was enlarged with increasing frequency from 0.1 to 10 Hz at 37°C. It was indicated that the formed hydrogel has decent gel strength which could sustain the drug release. Furthermore, T_1 and PPa in DLM-gel were stable under 10 mins laser irradiation (660 and 808 nm, 180 J/cm^2) which ensured that the fluorescence of PS no quenching during ex vivo/ in vivo monitoring. (Figure S3).

Figure 2. (a) SEM and TEM images of DML-gel, (inset) appearance of DLM-gel and F127 gel, **(b)** temperature ramp of gradient F127 concentrations, **(c)** frequency sweep of optimal formulation at 37C, G' and G'' represented elasticity modulus and viscosity modulus, individually, **(d)** release profile of free drugs, DLM and DLM-gel in PBS (pH 7.4), ($n=3$, error bars represent $\pm$ SD), **(e)** Δ Abs of ADPA in PBS, DLM, DLM-gel and gel-drug mixture irradiated with 808 nm laser for 10 min (300 J/cm^2 , $n=3$, error bars represent $\pm$ SD, $*p < 0.05$ compared to the control using student's t-test), (inset) Merged spectra of DLM-gel absorption change in 10 min.

Table 3. $T_{\text{sol-gel}}$, gelation time and viscosity of DLM-gel with the different F127 ratio. Results are expressed as mean values $\pm$ SD, ($n = 3$).

F127 (% w/w)	T _{sol-gel} (°C)	Gelation time (s) 37°C	Viscosity (mPa·s) 60rpm
14	N/A	N/A	
15	33.8 ± 0.3	45.1 ± 5.3	106.7 ± 3.8
16	29.7 ± 0.4	43.6 ± 2.3	115.1 ± 6.2
17	26.0 ± 0.3	38.1 ± 1.3	119.2 ± 7.3
18	22.4 ± 0.2	37.1 ± 4.5	131.1 ± 4.1
19	18.9 ± 0.3	32.1 ± 2.8	172.3 ± 5.7

Figure 2d showed the release profile of DLM-gel in PBS at 37°C. The cumulative release of both compounds in DLM-gel were less than 40% at 72 hours while the cargos in micelle solution were nearly 100% released. Both compounds were released at the similar rate which was favourable for energy transferring in TP-PDT. Such sustained release behaviour could be jointly contributed from micelle trapping and tight gel structures via hydrogen bonding in the aqueous solution. A ROS quencher, ADPA, was further applied to detect singlet oxygen ($^1\text{O}_2$) generation by monitoring the absorption bleaching at 400 nm. Accompanied with NIR light irradiation for 10 min, the absorption of DLM-gel was decreased by 0.21 ± 0.01 which is close to the drop value of DLM (**Figure 2e**). The similar degree of decrease was observed when DLM-gel was stored at 37°C for 3 days. On the contrary, simply mixing the same amount of both compounds into F127 hydrogels could hardly generate $^1\text{O}_2$ under the laser. It was reported that the water permeability of micelles could lead to a certain degree of PSs and two-photon compounds aggregation.⁴⁵ Consequently, the micelles not only prevented compounds from aggregating but also provided spatial distances less than 10nm between T1 and PPa for achieving FRET.

Cellular study. To investigate the cellular uptake, 4T1 cells were incubated with TPM-gel ($C_{\text{T1}} = 2.7 \mu\text{g/mL}$), PSM-gel ($C_{\text{PPa}} = 2.1 \mu\text{g/mL}$), DLM-gel ($C_{\text{T1}} = 2.7 \mu\text{g/mL}$, $C_{\text{PPa}} = 2.1 \mu\text{g/mL}$) and captured under two-photon confocal laser scanning microscopy (TP-CLSM). Both T1 and PPa in DLM-gel were well internalized with living cells, with the distribution of both compounds

primarily in the cytosol. (**Figure. 3a**) The overlaps between PPa and T1 ensured the short distance and facilitated the FRET process that is normally happened less than 10 nm.^{46,47} The cell viability assays with/without laser irradiation were applied to evaluate the cytotoxicity of DLM-gel. The cytotoxicity assays without light were performed on 4T1 murine breast cancer cells as cancerous model and human embryonic lung fibroblast (HEL F) cells which stood as normal cells. PPa, T1, DLM, DLM-gel, F127 and mPEG-PDLLA were showed non-significant cytotoxicity (above 80%) to both 4T1 and HEL F cells at their highest dosing concentrations. (**Figure S4**) This cytotoxicity study demonstrates that DLM-gel itself possesses low toxicity without light irradiation. To evaluate the *in vitro* anti-cancer effect of TP-PDT, 808 nm semiconductor laser was applied to irradiate 4T1 cells. The viability of DLM treated cells was decreased with prolonged irradiation time and increased laser intensity (**Figures. 3b** and **3c**). The cell viability was decreased to 25% with light dosage of 300 J/cm². The high efficiency of TP-PDT provided the foundation for inhibiting tumor growth *in vivo*. To demonstrate the *in situ* FRET process, the emission spectra of DLM-gel and TPM-gel locally on cells were extracted by using Lambda Stack scanning (Ex=808 nm). The characteristic emission peak of PPa at 683 nm appeared on the DLM-gel treated cells which pointing out the intracellular activation of the FRET process (**Figures 3d** and **3e**). *In situ* real-time TP-PDT was monitored by scanning DLM-gel treated cells under two-photon confocal microscopy with a 808 nm laser source for continued irradiation. Obivary morphology collapsing and bubbling was observed on the cell membrane and nucleus regions which is a characteristic phenomenon of PDT. (**Figures. 3f** inset and **3g**) The animation of real-time PDT was shown in electronic supporting materials (**Video S1**). The cell structure especially plasma membrane was damaged through the PDT process which could possibly associate with acute inflammation and anti-tumor immunity increasing. The cell morphology changes are also associated with ROS generation, which was in parallel detected by using 2',7'-dichlorofluorescein diacetate (DCFH-DA) as intracellular ROS probe. The increasing intensity of fluorescence again indicated that the rising level of intracellular ROS after NIR laser irradiating. (**Figure. 3h**)

Figure 3. (a) Two-photon microscope captured live 4T1 cells uptaking images which were treated with TPM-gel, PSM-gel and DLM-gel (T1, Ex= 808 nm, Em= 480 nm; PPa, Ex= 640 nm, Em= 710 nm), the scale bars represent 20 μ m. DLM-gel (C_{PPa} = 5.0 μ M, C_{T1} = 5.8 μ M) treated cells irradiated with gradient (b) time periods and (c) intensities, (n=6, error bars represent $\pm$ SD, *p < 0.05 and ** p < 0.01 compared to the control using student's t-test) Lambda stacks of (d) DLM and (e) TPM treated cells, the spectra acquired from the red circle position of the corresponding stack images on the left. The red arrow in represents the excitation of PPa by FRET effect. The scale bars represent 20 μ m, (f) Intracellular ROS level detection and (inset) cell collapsing, (g) cell morphology changes at numbered position '1, 2 and 3' before and after laser irradiation (808 nm, 300 J/cm², the scale bars represent 20 μ m) (h) quantification of DCFH-DA fluorescence intensity, ** p < 0.01.

***In vivo* and *ex vivo* distribution.** The *in vivo* and *ex vivo* distribution was investigated using BALB/c mice bearing 4T1 murine breast cancer subcutaneously implanted. The *in vivo* distribution results (**Figures 4a** and **4b**) showed that the injected DLM-gel formed *in situ* hydrogels (composed of 16% F127 with C_{PPa} 5.2 mg/kg and C_{T1} 4.9 mg/kg in mPEG-PDLLA micelles) within the tumor and the PPa fluorescence signal was considerably strong even at the 14th day post injection. DLM, oppositely, diffused through the tumor 24 hours after administration and cleared completely on the 4th day. The *ex vivo* distribution results confirmed the tumor accumulation of DLM-gel and it was hardly observed DLM-gel distributing PS in the rest of the organs by comparing with DLM group (**Figure. 4c**). PS fluorescence from *in vivo* and *ex vivo* drug distribution was initially distributed in the core of tumor and then permeate to entire tumor around 24 hours post injection. It was speculated that the intratumoral pressure could affect distribution of the formulation when it was in liquid state, and the compounds could then sustained release from the formed gel matrix. The long-term tumor retaining of the formulation could allow PS well uptaking by cancerous cells.

Furthermore, the tumor to liver ratio (TLR), a critical parameter to evaluate treatment response⁴⁸, of DLM-gel (**Figure 4d**) was calculated from fluorescence intensities of *ex vivo* organs

(**Figures 4e and 4f**) and it was significantly higher than DLM confirming further effective tumor retention. Both *in vivo* and *in vitro* release results, show that the two compounds retain in tumor, confirming that DLM is stabilizing PSs and facilitating *in vivo* FRET. Moreover, DLM solution was hardly injected more than 100 μL into tumor otherwise squeezing out by intratumoral pressure. In the contrary, formulation such as DLM-gel proved that this thermosensitive nanocomposite gel was suitable for intratumoral injection and retained in the lesion for comparatively long term. In clinical settings, patients need to avoid sun light exposing after PDT to precaution skin phototoxicity. DLM-gel intratumoral retention of the PSs bodes well to reduce light toxicity to skin. As a matter of fact, the elimination/metabolism of implanted high molecular weight F127 is concerned with the extravasation issue due to its non-biodegradable property.⁴⁹ We intended to use commercial available pharmaceutical excipients as DDS here for quality assurance and closer to a “medicine”. In the further study, PEO-PPO-PEO with lower molecular weight and conjugated with biodegradable liner will be carefully designed and push the two-photon PDT further into clinic.

***In vivo* anti-cancer efficacy.** The *in vivo* anti-cancer efficacy of DLM based TP-PDT was evaluated on the same animal models as above according to the administration regime detailed in **Table 1**. The ‘DLM-gel + laser’ group (TP-PDT) and ‘PSM-gel + laser’ group (One-photon PDT) irradiated by 808nm and 660nm laser irradiation (300 J/cm^2) respectively significantly inhibited tumor growth with corresponding tumor growth inhibition (TGI) rates of 67.3 % and 61.1 %. Similar anti-cancer efficacy of the two groups was speculated that the superficial location of tumor. The subcutaneous tumor model was different from large sized solid tumor in clinic and the advantageous deep tissue penetration of TP-PDT was not exhibited. The two treatments were compared again blocking the tumor with 1 cm thick swine muscle tissue. The TGI of one-photon PDT decreased down to 27.7%, while the TGI of TP-PDT group was still a respectable 53.8% even 21 days after injection. Furthermore, it was reported in a few cases that intratumoral injection of liquid preparation of PSs suspension showed non-significant anti-cancer efficacy due to limited

injection volume and low ROS yield.^{50,51} Result here demonstrates a superior tissue light penetration as well as a preparation preventing PS aggregation.

Meanwhile, there was no significant shifting in body weights between groups after the treatments. Histological analysis of the tumor sections were eventually analyzed by TUNEL staining, and the results were in agreement with the anti-cancer efficacy outcomes (**Figure 5e**) and TUNEL staining fluorescence quantification at 21st day after the first treatment was shown in **Figure S5**. The tissue blocked TP-PDT group induced a large population of cell apoptosis/necrosis. Furthermore, non-evident histological changes were observed in hematoxylin and eosin (H&E) stained tissue of all main organs after treated with DLM-gel (**Figure S6**). To preliminary investigate biocompatibility of the implant, 0.2 mL DLM-gel was injected into leg muscle, and histological analysis was carried out by H&E staining after 7 days. Comparing with the control group, non-significant changes were found which indicated that the *in situ* hydrogel nanocomposite possessing good biocompatibility (**Figure S7**). The decent anti-cancer efficacy of the nanocomposite gel based two-photon PDT was contributed by the localized retaining of drugs, deep therapeutic depth and ability of inducing large population of cancer cell apoptosis. In the potentially therapy process, firstly, cancer cell were blasted by the PDT treatment and the surrounding cells were induced apoptosis. In the meantime, the dead cells could release cytokines which stimulated local acute inflammation and increase anti-tumor immunity. These anti-tumor immune response could further assist the elimination of local and metastatic cancer cells.

Figure 5. *In vivo* anti-cancer efficacy: (a) tumor growth curves, black arrows indicate the time of injection and red arrows indicate the time of laser administration. (n=6, error bars represent $\pm$ SD, *p < 0.05, ** p < 0.01, compared to the control group using student's t-test); (b) tumor growth inhibition rates; (c) tumor growth curves of 1 cm tissue blocked PDT, (n=6, error bars represent $\pm$ SD, *p < 0.05 compared to the control group using student's t-test) (d) mice body weight shifting, (e) Merged images of TUNEL stained tumor slides showing the induced apoptosis in green colour.

Conclusions

In summary, the mPEG-PDLLA micelles and F127 thermosensitive hydrogel composite based deep tissue TP-PDT showed tumor specific retaining and strong anti-cancer efficacy against mouse breast cancer. The drug loaded micelles and hydrogel jointly promoted FRET efficiency and prevented PSs from aggregating. DLM-gel was convenient to administrate due to the low viscosity below room temperature and could be long-term accumulated in the tumor after intra-tumoral administrated rather than distributed in normal organs. Significantly, the TP-PDT was activated by NIR laser and generated massive therapeutic ROS in deep tissue which significantly inhibited the tumor growth. Moreover, DLM-gel showed non-significant systemic toxicity and good biocompatibility. Such composite DDS with decent efficacy and biocompatibility shows great potential for cancer therapy.

Declarations of interest: none

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5