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An Organic Afterglow Pro-theranostic Nanoassembly

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Abstract: Cancer theranostics holds potential promise for precision medicine; however, most existing theranostic nanoagents are simply developed by doping both therapeutic agents and imaging agent into one particle entity, and thus have “always-on” pharmaceutical effect and imaging signals regardless of their in vivo location. Herein, we report the development of an organic afterglow pro-theranostic nanoassembly (APtN) that specifically activates both pharmaceutical effect and diagnostic signals in response to a tumor-associated chemical mediator (hydrogen peroxide, H_2O_2). APtN comprises an amphiphilic macromolecule and a near-infrared (NIR) dye acting as the H_2O_2 -responsive afterglow pro-drug and the afterglow initiator, respectively. Such a molecular architecture allows APtN to passively target the tumor in living mice, specifically release the anticancer drug in the tumor, and spontaneously generate the uncaged afterglow substrate. Upon near-infrared (NIR) light pre-irradiation, the afterglow initiator generates singlet oxygen to react and subsequently transform the uncaged afterglow substrate into an active self-luminescent form. Thus, the intensity of generated afterglow luminescence is correlated with the drug release status, permitting real-time in vivo monitoring of pro-drug activation. This study proposes a background-free design strategy towards activatable cancer theranostics.

Keywords: optical imaging, cancer theranostics, activatable probes, organic nanoparticles

Cancer theranostics integrates therapeutic action and diagnostic ability into one entity, providing multimodal abilities for early detection of diseases, targeted drug delivery and real-time tracking of therapeutic outcome.^[1] An ideal cancer theranostic system should only release therapeutic agent and activate its diagnostic signal in response to the intended cancer biomarkers.^[2] However, most existing theranostic nanoagents are simply composed of both therapeutic agents and imaging agent in one nanoparticle entity, and their pharmaceutical effect and imaging signals are always on regardless of their location in living subjects.^[3] There are only a few of existing theranostic systems that can activate the biomarker-specific signal with close correlation with drug release status, and they unfortunately utilize fluorescence as the signal readout.^[4] Because of strong tissue autofluorescence accompanied with light excitation, such fluorescent theranostic systems have poor imaging sensitivity for disease detection and minoring of drug release.^[5]

Excitation-free luminescence imaging including persistent/afterglow luminescence and chemiluminescence imaging is a background-minimalized optical modality.^[6] Such luminescence imaging often utilizes optical agents that can slowly release photons under certain physiological conditions, enabling detection of signals in the absence of light excitation.^[7] Thereby, it has significantly higher signal-to-background ratio and deeper tissue penetration than fluorescence imaging.^[8] Despite its potential in cancer theranostics, there are only few kinds of water-soluble afterglow materials with the ability to emit light after cessation of light excitation.^[9] Recently, we found that poly(para-phenylenevinylene] (PPV)-based nanoparticles could emit afterglow luminescence, and its mechanism is related to the in-situ production of a chemiluminescent intermediate (1,2-dioxetane) upon light irradiation. Such chemical defects spontaneously degrade to release photons as one of its products.^[7, 10] PPV-based afterglow luminescence has been exploited for activatable detection of drug-induced acute liver injury and real-time monitoring of photothermal temperatures, showing its advantages over fluorescence in in vivo imaging.^[7, 11]

In this study, we report the synthesis of an organic afterglow pro-theranostic nanoassembly (APtN) that specifically activates both pharmaceutical effect and diagnostic signals in the tumor microenvironment (TME) of living mice. APtN is self-assembled from an amphiphilic macromolecule (PEG-AE-5-DFUR) and a near infrared (NIR) dye (silicon 2,3-naphthalocyanine bis(trihexylsilyloxy): NCBS) (Figure 1a), which act as the afterglow pro-drug and the afterglow initiator, respectively. In addition to the hydrophilic PEG tail, PEG-AE-5DFUR has a hydrophobic head, which is composed of an afterglow substrate (adamantylidene-enol ether) conjugated with a prodrug (5'-deoxy-5-fluorouridine: 5DFUR) via a hydrogen peroxide (H_2O_2) cleavable linker (phenylboronic ester group).^[12] 5DFUR, a prodrug of 5-fluorouracil, has been used clinically in the treatment of different cancers.^[13] Such a molecular design allows APtN to passively target tumor after intravenously injection via enhanced permeability and retention (EPR) effect (Figure 1b).^[14] After accumulation in TME, the phenylboronic ester group of APtN is specifically cleaved by the tumor-upregulated H_2O_2 ,^[15] consequently releasing 5DFUR and generating PEG-AE with the uncage afterglow substrate (adamantylidene-enol ether) (Figure 1c). Upon NIR laser irradiation, the NCBS of APtN serves as the afterglow initiator to generate $^1\text{O}_2$ to react with the uncage afterglow substrate, leading to the formation of self-luminescent substrate (PEG-dioxetane).^[16] In such a way, not only the theranostic responses (pharmaceutical action & detection signal) are triggered by a cancer biomarker, but also the afterglow signal is correlated with the status of drug release.

Figure 1. Design and proposed molecular mechanism of APtN for cancer theranostics. a) Synthesis of APtN and the chemical structures of the afterglow pro-drug (PEG-AE-5-DFUR) and the afterglow initiator (NCBS). Schematic illustration b) and detailed structure conversion c) H_2O_2 -induced activation of pro-drug and afterglow luminescence for APtN.

The amphiphilic macromolecule (PEG-AE-5-DFUR) was synthesized according to Scheme 1. An adamantylidene-enol ether (compound 1),^[17] possessing excitation free luminescence imaging ability after reacted with singlet oxygen ($^1\text{O}_2$) to form dioxetane, was selected as the hydrophobic segment to conjugate with 5DFUR and connected to hydrophilic PEG. First, compound 1 was reacted with trifluoromethanesulfonic anhydride to give compound 2, followed by reaction with bis(pinacolato)diboron, potassium acetate and [1,1'-bis(diphenylphosphino) ferrocene] dichloropalladium(II) to form compound 3, and further treatment with NaOH to afford compound 4. Compound 4 was then reacted with 3-azido-1-propanol to form compound 5 through condensation reaction. The chemical structures of these compounds were validated by proton nuclear magnetic resonance (^1H NMR) spectra and liquid chromatography–mass spectra (LC-MS) (Figure S1 and S2,

Supporting Information). To synthesize amphiphilic PEG-adamantylidene-enol ether boronic acid, termed as PEG-AE, compound 5 was reacted with PEG-alkyne by copper(I)-catalyzed alkyne-azide cycloaddition reaction in the presence of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and sodium ascorbate. Finally, PEG-AE was reacted with 5DFUR by ester condensation reaction between phenylboronic acid and vicinal diol to form PEG-AE-5DFUR. The structure of PEG-AE-5DFUR was verified by ^1H NMR, showing the specific peaks of triazole proton (7.8 ppm) and methylene protons of phenylboronic pinacol ester group (4.28, 3.65 ppm) (Figure S1, Supporting Information).

Scheme 1. Synthetic routes of the afterglow pro-drug (PEG-AE-5-DFUR). Reagents and conditions: (i) pyridine, trifluoromethanesulfonic anhydride, anhydrous dichloromethane (DCM), 25 °C, 1 h; (ii) potassium acetate, bis(pinacolato)diboron, [1,1'-Bis(diphenylphosphino) ferrocene] dichloropalladium(II), dried Dioxane, 120 °C, 3 h; (iii) NaOH solution (1 mM), tetrahydrofuran (THF), 25 °C, 8 h; (iv) 3-azido-1-propanol, N,N'-dicyclohexylcarbodiimide (DCC), 4-Dimethylaminopyridine (DMAP), 25 °C, 24 h; (v) PEG-alkyne, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, sodium ascorbate, water, dimethyl sulfoxide (DMSO), 25 °C, 24 h; (vi) 5'-deoxy-5-fluorouridine (5DFUR), dried THF, molecular sieves, 25 °C, 24 h.

The amphiphilicity of PEG-AE-5-DFUR allowed it to directly self-assemble and encapsulate the NIR dye (NCBS) into homogenous nanoparticles in aqueous media, affording APtN.^[18] According to the absorption of NCBS at 770 nm, the doping amount of NCBS was optimized at 2.8w/w%. (Figure S3a, Supporting Information). As measured by transmission electron microscopy (TEM) and

dynamic light scattering (DLS) (Figure 2a), APtN had uniform spherical nanostructure with an average hydrodynamic diameter of (55 ± 5) nm and a relatively narrow size distribution (polydispersity = 0.17). In addition, after storage at 37 °C for 20 days both in PBS and 10% FBS solution or dilution, APtN maintained its initial morphology and showed negligible size change (Figure S3, Supporting Information), indicating its high aqueous stability.

The response of APtN towards H_2O_2 was tested in PBS under physiological conditions.^[19] In the absence of H_2O_2 , APtN has the fluorescence emission at 820 nm due to the existence of NCBS, but nearly undetectable afterglow luminescence after light pre-irradiation. After treatment with H_2O_2 , the hydrodynamic diameter of APtN decreased from 55 ± 5 to 24 ± 3 nm (Figure 2a). The NIR fluorescence remained the same after H_2O_2 treatment (Figure 2c and S4, Supporting Information), because NIR was inert to H_2O_2 . However, afterglow luminescence was detected with the maximum at 550 nm (Figure 2b), which was 820 times higher than that without H_2O_2 treatment. The afterglow of APtN had a half-life of 16 min (Figure 2d), and could be visualized by naked eye and captured by a digital camera at a relatively high concentration (20 mg/mL) (Figure S5 in the Supporting Information). APtN had a low Limit of detection (LOD) to H_2O_2 (100 nM) (Figure 2e). More importantly, the afterglow of APtN had a linear response with increased $[\text{H}_2\text{O}_2]$ (Figure S6a,b, Supporting Information), and was only activated by H_2O_2 not other ROS including $\text{OH}^\bullet$, OCl^- , ONOO^- and $\text{O}_2^{\bullet-}$ (Figure 2f and Figure S6, Supporting Information). These data proved that APtN had sensitive and specific afterglow response towards to H_2O_2 .

The mechanism of specific H_2O_2 responses along with the drug release kinetics of APtN was further studied by high performance liquid chromatography (HPLC) analysis. After treatment with H_2O_2 , the new elution peaks were observed at 16.3 and 3.4 min, corresponding to PEG-adamantylidene-enol ether and 5-DFUR, respectively. This approved the H_2O_2 -induced release of 5DFUR from APtN. (Figure 2g). Besides, APtN showed similar drug release kinetics to PEG-AE-5-DFUR (Figure 2h and

S6c, Supporting Information), showing 60 and 90% releases of 5DFUR at 4 and 24 h post-treatment with H_2O_2 at TME-mimicking acidic condition ($\text{pH}=6.0$), respectively. Such release profile was faster than that at normal pH with or without H_2O_2 . The afterglow intensity was linearly correlated with the released percentage of 5DFUR with R^2 as 0.9857 (Figure 2i); moreover, it increased with increased [APtN] (Figure S6d, Supporting Information). These data suggested that H_2O_2 -activated afterglow of APtN could be used for real-time monitoring of drug activation and release.

Figure 2. In vitro characterization of APtN in response to H_2O_2 . a) Representative DLS and TEM images of APtN before and after H_2O_2 treatment. Afterglow luminescence b) and Fluorescence spectra c) of APtN in the absence or presence of H_2O_2 (100 μM , 37 $^\circ\text{C}$, 0.5 h) in PBS ($\text{pH} = 7.4$). Afterglow luminescence spectra was acquired with IVIS spectrum imaging system with different specific emission filters. Inset: b) the corresponding afterglow images and c) fluorescence images

acquired with IVIS spectrum imaging system. d) Decay of afterglow luminescence of APtN (1mg/mL) in PBS (pH = 7.4) at room temperature. The solution was pretreated with H₂O₂ (100 μ M, 37 $^{\circ}$ C, 0.5 h) and pre-irradiated for 10 s with NIR light (808 nm) at a power density of 0.25 W/cm² before detection by bioluminescence mode of IVIS spectrum imaging system with open filter. e) Afterglow intensities of APtN (1 mg/mL) in PBS (pH = 7.4) treated with different concentrations of H₂O₂. The afterglow signals were measured by GloMax[®] Luminometer. f) Afterglow intensities of APtN measured by GloMax[®] Luminometer in the presence of different ROS at a concentration of 100 μ M over 0.5 h. Insert: the corresponding afterglow images acquired under bioluminescence mode of IVIS spectrum imaging system. g) HPLC traces of 5-DFUR (black line), APtN in the absence (red line) or presence (blue line) of H₂O₂ (100 μ M, 37 $^{\circ}$ C, 0.5 h). Wavelength: 254 nm. h) 5DFUR release profiles of APtN in PBS (pH = 6.0) and PBS (pH = 7.4) in the absence or presence of H₂O₂ (100 μ M, 37 $^{\circ}$ C). i) Correlation between the afterglow luminescence of APtN acquired with IVIS spectrum imaging system and the 5DFUR release percentage determined by HPLC. ***p < 0.001.

The H₂O₂-activated theranostic response of APtN was tested in living mouse breast (4T1) cancer cells. Note that PEG-AE caused negligible cytotoxicity against 4T1 cells but the cytotoxicity of APtN was obviously enhanced by addition of exogenous H₂O₂ (Figure S7a&S7b, Supporting Information). 4T1 cancer cells were pre-treated with a cell-permeable antioxidant (N-acetylcysteine: NAC),^[20] or a γ -glutamyl cysteine synthase inhibitor (D,L-Buthionine-(S,R)-sulphoximine: BSO).^[21] After incubation with APtN for 4 h, similar strong NIR fluorescence signal was observed in 4T1 cells (Figure 3a) for the control, BSO pre-treated and NAC pre-treated cells (Figure 3c). This indicated that the cellular uptake of APtN was similar for three treatments. After NIR light pre-irradiation (note that such a short period light irradiation had neglectable cytotoxicity, Figure S7c, Supporting Information), bright afterglow luminescence was observed for BSO pre-treated cells (Figure 3b and S7d, Supporting Information), which was 5.9-fold and 2.2-fold higher than that of NAC pre-treated and control groups, respectively (Figure 3d and S7e Supporting Information). This should be due to the fact that BSO inhibited γ -glutamyl cysteine synthase, an enzyme important for glutathione biosynthetic pathway, which subsequently depleted glutathione and increased cellular ROS level

(Figure 3e). In contrast, NAC scavenged reactive oxygen intermediates, preventing oxidative stress and subsequently decreasing cellular H_2O_2 level. These data validate that the afterglow of APtN could be activated by endogenous H_2O_2 .

Figure 3. In vitro afterglow imaging of APtN in 4T1 cells. Fluorescence (a) and afterglow (b) images and corresponding average fluorescence (c) and afterglow intensities (d) of 4T1 cells captured from confocal laser scanning microscope (CLSM) and inverted fluorescence microscope, respectively. e) Schematic illustration of the effect of pre-treatments with NAC or BSO on the afterglow intensities of APtN in cells. Cells were pre-treated with saline, BSO (20 μM) and NAC (20 μM) for 2 h, then replaced with fresh medium containing APtN (2 mg/mL), incubated for 4 h and replaced with fresh medium. For afterglow imaging, cells were pre-irradiated with NIR light (808 nm) at a power density of 0.25 W/cm^2 for 10 s, and images were captured from inverted fluorescence microscope at an exposure time of 30 s. *** $p < 0.001$

The feasibility of APtN for cancer theranostics was validated in xenograft 4T1 tumor mouse model. Mice were pre-treated with BSO or NAC followed by intravenous injection of APtN (Figure 4a). As the NIR fluorescence of APtN was a constant feature, real-time NIR fluorescence imaging was conducted to monitor and qualify the accumulation of APtN in the tumor of living mice (Figure 4b and S8a, Supporting Information). The NIR fluorescence at tumor sites gradually increased and reached the similar maximum at 24 h post-injection of APtN for the three groups (control, BSO pre-treated and NAC pre-treated). At this timepoint, the NIR fluorescence was ~16 times higher than the background, indicating that APtN could effectively accumulate into tumor site via EPR effect.^[22] To evaluate drug release status, afterglow imaging was conducted by pre-irradiation of living mice with NIR laser for 20 s. The afterglow signals could be detected for all groups (Figure 4c), showing 38 (control group), 96 (BSO pre-treated group) and 29 (NAC pre-treated group) times higher intensity than the background. However, BSO pre-treated group had the strongest brightness, which was 3.5-fold and 2-fold higher than that observed from NAC pre-treated and control groups, respectively (Figure 4d). The NIR fluorescence signals of excised tumor tissues especially at 24 h post-injection of APtN were similar for the three groups, while the afterglow signal of BSO pre-treated tumor tissue was 2.54 and 1.65 times higher than NAC pre-treated and control groups, respectively (Figure S8 and S9, Supporting Information). The difference in the afterglow signals of these groups was consistent with the *in vitro* data, which was attributed to the different H₂O₂ concentrations in the TME mediated by the treatment of the ROS scavenger (NAC) and the glutathione biosynthesis inhibitor (BSO). In addition, the *ex-vivo* data (Figure 4e) showed the similar enhancement trend to *in vivo* imaging, further validating the utility of APtN for monitoring of H₂O₂-induced drug release. Note that no obvious tissue damage was observed in the histological hematoxylin and eosin (H&E) staining of main organs of mice after injection of APtN (Figure S10, Supporting Information), implying its good biocompatibility.

Figure 4. In vivo afterglow imaging of APtN. a) Schematic illustration of the treatment timeline for drug treatment and real-time imaging. Mice of different groups were first treated with saline, BSO (30 mg/mL, 0.2 mL/day) or NAC (30 mg/mL, 0.2 mL/day) through the tail vein for two days, and then APtN (400.0 μ g/mL, 0.2 mL) was injected intravenously for real-time imaging. For afterglow imaging, tumor sites were pre-irradiated with NIR light (808 nm) at a power density of 0.25 W/cm² for 20 s before detection. b) In vivo NIR fluorescence intensities of 4T1 tumor in living mice at different timepoints after intravenous injection of APtN. NIR fluorescence imaging and afterglow images c) and average fluorescence and afterglow intensities d) of 4T1 tumor in living mice after intravenous injection of APtN for 24 h. The 4T1 tumor was indicated by the white dashed circles. ***p < 0.001

In summary, we have developed an organic afterglow pro-theranostic nanoassembly (APtN) that can specifically activate both pharmaceutical drug and diagnostic afterglow signals in TME by a tumor-associated chemical mediator (H₂O₂) for cancer theranostics. The afterglow of APtN could be enhanced by 820-fold in solution after treatment with H₂O₂, and the signal intensity was correlated well with the released amount of the drug (5DFUR). Such H₂O₂-induced pro-drug release and afterglow enhancement of APtN was further validated in both cells and mouse model. The results

consistently showed that the afterglow signal was enhanced in the elevated ROS environment, providing the real-time feedback for the status of pro-drug activation. In view of the small size (55 nm) and ideal biodistribution, APtN holds great promise for cancer therapy. Thus, this study not only presents the first excitation-free nanoagent that enables diseases-activated drug releases and signal correlation, but also provides design guidelines towards smart cancer theranostics.

Experimental Section

The experimental details are provided in the Supporting Information. All mouse experimental procedures were approved by the Institutional Animal Care and Use Committee, Nanyang Technological University (IACUC, NTU) (Approval No. V05-20180911).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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An organic afterglow pro-theranostic nanoassembly (APtN) is developed to specifically activate both pharmaceutical drug and diagnostic afterglow signals in the tumor microenvironment by a tumor-associated chemical mediator (H_2O_2) for cancer theranostics.

Keywords: optical imaging, cancer theranostics, activatable probes, organic nanoparticles

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