An Organic Afterglow Protheranostic Nanoassembly

Shasha He, Chen Xie, Yuyan Jiang, and Kanyi Pu*

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 an “always-on” pharmaceutical effect and imaging signals regardless of their in vivo location. Herein, the development of an organic afterglow protheranostic nanoassembly (APtN) that specifically activates both the pharmaceutical effect and diagnostic signals in response to a tumor-associated chemical mediator (hydrogen peroxide, H$_2$O$_2$) is reported. APtN comprises an amphiphilic macro-molecule and a near-infrared (NIR) dye acting as the H$_2$O$_2$-responsive afterglow prodrug and the afterglow initiator, respectively. Such a molecular architecture allows APtN to passively target tumors in living mice, specifically release the anticancer drug in the tumor, and spontaneously generate the uncaged afterglow substrate. Upon NIR light preirradiation, 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 prodrug activation. This study proposes a background-free design strategy toward activatable cancer theranostics.

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 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 monitoring of drug release.[5]

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]

Excitation-free luminescence imaging including persistent/afterglow luminescence and chemiluminescence imaging is a background-minimized 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(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]

In this study, we report the synthesis of an organic afterglow protheranostic 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(trihexylsilyloxide): NCBS) (Figure 1a), which act as the afterglow prodrug and the afterglow initiator, respectively. In addition to the hydrophilic PEG tail, PEG-AE-5-DFUR has a hydrophobic head, which is composed of an afterglow substrate (adamantylidene-enol ether) conjugated with a prodrug (5′-deoxy-5-fluorouridine, 5-DFUR) via a hydrogen peroxide (H$_2$O$_2$) 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$_2$O$_2$,[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 O$_2$ to react with the uncage afterglow substrate, leading to the formation of

![Image](https://www.advmat.de)

Dr. S. He, Dr. C. Xie, Y. Jiang, Prof. K. Pu
School of Chemical and Biomedical Engineering
Nanyang Technological University
70 Nanyang Drive, Singapore 637457, Singapore
E-mail: kypu@ntu.edu.sg

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/adma.201902672.

DOI: 10.1002/adma.201902672
self-luminescent substrate (PEG-dioxetane).\textsuperscript{[16]} In such a way, not only the theranostic responses (pharmacetical action and detection signal) are triggered by a cancer biomarker, but also the afterglow signal is correlated with the status of drug release.

The amphiphilic macromolecule (PEG-AE-5-DFUR) was synthesized according to Scheme 1. An adamantylidene-enol ether (compound 1),\textsuperscript{[17]} possessing excitation free luminescence imaging ability after reacted with singlet oxygen (\textsuperscript{1}O\textsubscript{2}) to form dioxetane, was selected as the hydrophobic segment to conjugate with 5-DFUR 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 (\textsuperscript{1}H NMR) spectra and liquid chromatography–mass spectra (LC–MS) (Figures S1 and S2, Supporting Information). To

Figure 1. Design and proposed molecular mechanism of APtN for cancer theranostics. a) Synthesis of APtN and the chemical structures of the afterglow prodrug (PEG-AE-5-DFUR) and the afterglow initiator (NCBS). b,c) Schematic illustration (b) and detailed structure conversion (c) H\textsubscript{2}O\textsubscript{2}-induced activation of prodrug and afterglow luminescence for APtN.

Scheme 1. Synthetic routes of the afterglow prodrug (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 × 10\textsuperscript{−3} M), 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, CuSO\textsubscript{4} \cdot 5H\textsubscript{2}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.
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 CuSO$_4$·5H$_2$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-5-DFUR. The structure of PEG-AE-5-DFUR was verified by $^1$H 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).

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 2.8 w/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 $(55 \pm 5)$ 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% fetal bovine serum (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 toward H₂O₂ was tested in PBS under physiological conditions.[19] In the absence of H₂O₂, APTN has the fluorescence emission at 820 nm due to the existence of NCBS, but nearly undetectable afterglow luminescence after light preirradiation. After treatment with H₂O₂, the hydrodynamic diameter of APTN decreased from 55 ± 5 to 24 ± 3 nm (Figure 2a). The NIR fluorescence remained the same after H₂O₂ treatment (Figure 2c; Figure S4, Supporting Information), because NIR was inert to H₂O₂. However, afterglow luminescence was detected with the maximum at 550 nm (Figure 2b), which was 820 times higher than that without H₂O₂ 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, however only at a relatively high concentration (20 mg mL⁻¹) (Figure S5 in the Supporting Information). APTN had a low Limit of detection (LOD) to H₂O₂ (100 × 10⁻⁹ M) (Figure 2e). More importantly, the afterglow of APTN had a linear response with increased [H₂O₂] (Figure S6a,b, Supporting Information), and was only activated by H₂O₂ not other reactive oxygen species (ROS) including OH⁻, OCl⁻, ONOO⁻, and O₂⁻ (Figure 2f; Figure S6, Supporting Information). These data proved that APTN had sensitive and specific afterglow response toward H₂O₂.

The mechanism of specific H₂O₂ responses along with the drug release kinetics of APTN was further studied by high performance liquid chromatography (HPLC) analysis. After treatment with H₂O₂, 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 H₂O₂-induced release of 5DFUR from APTN. (Figure 2g). Besides, APTN showed similar drug release kinetics to PEG-AE-5-DFUR (Figure 2h; Figure S6c, Supporting Information), showing 60 and 90% releases of 5-DFUR at 4 and 24 h post-treatment with H₂O₂ at TME-mimicking acidic condition (pH = 6.0), respectively. Such release profile was faster than that at normal pH with or without H₂O₂. The afterglow intensity was linearly correlated with the released percentage of 5DFUR with R² as

Figure 3. In vitro afterglow imaging of APTN in 4T1 cells. a–d) Fluorescence (a) and afterglow (b) images and corresponding average fluorescence (c) and afterglow intensities (d) of 4T1 cells captured from confocal laser scanning microscopy (CLSM) and inverted fluorescence microscopy, respectively. e) Schematic illustration of the effect of pretreatments with NAC or BSO on the afterglow intensities of APTN in cells. Cells were pretreated with saline, BSO (20 × 10⁻⁶ M), and NAC (20 × 10⁻⁶ M) for 2 h, then replaced with fresh medium containing APTN (2 mg mL⁻¹), incubated for 4 h and replaced with fresh medium. For afterglow imaging, cells were preirradiated 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 microscopy at an exposure time of 30 s. ***p < 0.001.
0.9857 (Figure 2i); moreover, it increased with increased [APtN] (Figure S6d, Supporting Information). These data suggested that H₂O₂-activated afterglow of APtN could be used for real-time monitoring of drug activation and release.

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,b, Supporting Information). 4T1 cancer cells were pretreated with a cell-permeable antioxidant (N-acetyl-cysteine: 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 pretreated and NAC pretreated cells (Figure 3c). This indicated that the cellular uptake of APtN was similar for three treatments. After NIR light preirradiation (note that such a short period light irradiation had negligible cytotoxicity, Figure S7c, Supporting Information), bright afterglow luminescence was observed for BSO pretreated cells (Figure 3b; Figure S7d, Supporting Information), which was 5.9-fold and 2.2-fold higher than that of NAC pretreated and control groups, respectively (Figure 3d; Figure 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 H₂O₂ level. These data validate that the afterglow of APtN could be activated by endogenous H₂O₂.

The feasibility of APtN for cancer theranostics was validated in xenograft 4T1 tumor mouse model. Mice were pretreated with BSO or NAC followed by intravenously 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 quantify the accumulation of APtN in the tumor of living mice (Figure 4b; Figure S8a, Supporting Information). The NIR fluorescence at tumor sites gradually increased and reached the similar maximum at 24 h postinjection of APtN for the three groups (control, BSO pretreated, and NAC pretreated). At this time point, 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 preirradiation 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 pretreated group), and 29 (NAC pretreated group) times higher intensity than the background. However, BSO pretreated group had the strongest brightness, which was 3.5-fold and twofold higher than that observed from NAC pretreated and control groups, respectively (Figure 4d). The NIR fluorescence signals of excised tumor tissues especially at 24 h postinjection of APtN were similar for the three groups, while the afterglow signal of

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⁻¹, 0.2 mL day⁻¹), or NAC (30 mg mL⁻¹, 0.2 mL day⁻¹) through the tail vein for two days, and then APtN (400.0 µg mL⁻¹, 0.2 mL) was injected intravenously for real-time imaging. For afterglow imaging, tumor sites were preirradiated with NIR light (808 nm) at a power density of 0.25 W cm⁻² 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. c,d) 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.
BSO pretreated tumor tissue was 2.54 and 1.65 times higher than NAC pretreated and control groups, respectively (Figures 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 H$_2$O$_2$ concentrations in the TME mediated by the treatment of the ROS scavenger (NAC) and the glutathione biosynthesis inhibitor (BSO). In additional, the ex vivo data (Figure 4e) showed the similar enhancement trend to in vivo imaging, further validating the utility of APtN for monitoring of H$_2$O$_2$-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.

In summary, we have developed an organic APtN that can specifically activate both pharmaceutical drug and diagnostic afterglow signals in TME by a tumor-associated chemical mediator (H$_2$O$_2$) for cancer theranostics. The afterglow of APtN could be enhanced by 820-fold in solution after treatment with H$_2$O$_2$, and the signal intensity was correlated well with the released amount of the drug (5DFUR). Such H$_2$O$_2$-induced prodrug 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 prodrug 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 nanosensor that enables diseases-activated drug releases and signal correlation, but also provides design guidelines toward smart cancer theranostics.

Experimental Section

The experimental details are provided in the Supporting Information.

Supporting Information

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

Acknowledgements

This work was supported by Nanyang Technological University (NTU-SUG: M4081627) and Singapore Ministry of Education Academic Research Fund Tier 1 (RG133/15 M4011559, 2017-T1-002-134-RG147/17) and Tier 2 (MOE2016-T2-1-098). All mouse experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC), Nanyang Technological University (NTU).

Conflict of Interest

The authors declare no conflict of interest.

Keywords

activatable probes, cancer theranostics, optical imaging, organic nanoparticles

Received: April 26, 2019
Revised: June 3, 2019
Published online:


