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Semiconducting Polymer Nanoenzymes with Photothermic Activity for Enhanced Cancer Therapy

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Abstract: Regulation of enzyme activity is fundamentally challenging but practically meaningful for biology and medicine. However, noninvasive remote control of enzyme activity in living systems has been rarely demonstrated and exploited for therapy. Herein we synthesize a semiconducting polymer nanoenzyme with photothermic activity to digest collagen for enhanced cancer therapy. Upon near-infrared (NIR) light irradiation, the activity of the nanoenzyme can be enhanced by 3.5-fold to efficiently digest collagen in tumor extracellular matrix (ECM), leading to enhanced nanoparticle accumulation in tumors and consequently improved photothermal therapy (PTT). This study thus provides a promising strategy to remotely regulate enzyme activity for cancer therapy.

Controlling of enzyme activity in living systems at designated locations and times helps understand their underlying physiological functions and potentially could lead to new medicines.^[1] However, there are only a few approaches to remotely regulate enzyme activity. For instance, chemical modification of enzyme using biomarker-responsive segments presents a way to reversibly deactivate and restore enzyme activity.^[2] The use of conditional protein splicing system is another way to generate functional enzymes from inactive fragments in the presence of ligands.^[3] Local hyperthermia under an alternating magnetic field has also been used to increase the activity of thermophilic enzymes for biocatalysis.^[4] In contrast to these approaches, light activation seems to be a more ideal noninvasive method to control enzyme activity because of its simpler operability, better controllability and higher spatiotemporal resolution.^[5] Currently, light activation of enzymes is generally relied on ultraviolet (UV) and visible light,^[6] which has shallow tissue penetration and thus limited in vivo applications.^[7]

Semiconducting polymer nanoparticles (SPNs) with controllable optical properties have been exploited for optical imaging and phototherapy.^[8] Particularly, they can efficiently convert light into heat for photothermal therapy (PTT) and photoacoustic (PA) imaging.^[9] In addition, the high photothermal ability of SPNs allows them to serve as nanotransducers to remotely control gene expression^[10] and thermosensitive ion channels in living systems.^[11] However, such photothermal feature of SPNs has not been exploited to control enzyme activity so far.

In this study, we report the synthesis of semiconducting polymer nanoenzymes with near-infrared (NIR) photothermic activity and demonstrate their proof-of-concept application in in-situ and on-demand activation for enhanced cancer therapy in

living mice. Such nanoenzyme contains two key components: semiconducting polymer amphiphile and bromelain (Bro), serving as photothermal nanotransducer and temperature-sensitive enzyme, respectively. Bro ($M_w = 33000$) is a protease that proficiently digests collagen, and its optimal activity is $\sim 45^\circ\text{C}$.^[12] Thus, under NIR laser irradiation, the semiconducting polymer nanoenzymes can undergo efficient photothermal conversion (**Figure 1**), leading to the local temperature increase and thus the photothermic activation of collagen digestion. As collagen is the most abundant tumor extracellular matrix (ECM) protein,^[13] such a photothermally-triggered collagen digestion can enhance the accumulation of nanoparticles in the tumor, enabling improved PTT.

Figure 1. Mechanistic illustration of photothermally triggered enzyme activation of PCB1-Bro towards collagen digestion for enhanced accumulation of nanoparticles in tumor.

To construct the semiconducting polymer nanoenzymes, two semiconducting polymer amphiphiles (PCB1 and PCB2) were synthesized via a grafting-on approach (**Table S1** and **Figures S1-2**, Supporting Information). PCB1 was grafted with the short chain methoxy-polyethylene glycol (PEG) (M_w , 1000) and the long chain carboxyl-PEG (M_w , 2000); whereas, PCB2 was grafted with both long chain methoxy-PEG (M_w , 2000) and long chain carboxyl-PEG (M_w , 2000). The percentage of carboxyl-PEG in both PCB1 and PCB2 was optimized to 20%. The resulted semiconducting polymer amphiphiles were further covalently linked to Bro (**Figure 2a**), affording PCB-Bro. Bradford protein assay revealed that each PCB1 particle had approximately 4 enzyme molecules with the enzyme/PCB weight ratio of 73.2% (the coupling efficiency of 14.6%); in contrast, the weight ratio was much low ($< 10\%$) for PCB2. This should be attributed to the steric hindrance as the carboxyl groups were at the end of PEG with the length identical to those methoxyl-PEG for PCB2. Therefore, PCB1-Bro was used in the following studies.

After bioconjugation reaction, PCB1-Bro displayed a much less migration than PCB1 in the agarose gel electrophoresis (**Figure 2b**). Transmission electron microscopy (TEM) image showed that PCB1-Bro had a spherical morphology with a narrow size distribution (**Figure 2c**). The hydrodynamic diameter was increased from 18.5 ± 3.5 nm for PCB1 to 27.3 ± 2.2 nm for PCB1-Bro (**Figure 2d**). In addition, the zeta potential of PCB1-Bro (-9.3 ± 0.7 mV) was apparently increased relative to that of

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PCB1 (-12.5 ± 0.6 mV) (Figure S3, Supporting Information). Furthermore, both PCB1 and PCB1-Bro had good colloidal stability in phosphate buffered saline (PBS) for at least 2 weeks with no obvious changes in the hydrodynamic diameter (Figure S4, Supporting Information).

Figure 2. (a) Chemical structures of PCB1 and PCB2 and schematic for the synthesis of PCB-Bro. (b) Agarose gel electrophoresis of PCB1 and PCB1-Bro. (c) Representative TEM image of PCB1-Bro. (d) Dynamic light scattering profiles of PCB1 and PCB1-Bro. (e) UV-vis absorption and fluorescence spectra of PCB1 and PCB1-Bro. (f) Temperature increase of PBS, Bro, PCB1 and PCB1-Bro as a function of laser irradiating time. Inserts are the thermal images of PBS (1), Bro (2), PCB1 (3) and PCB1-Bro (4) at their respective maximum temperatures.

The optical and photothermal properties of PCB1-Bro were studied and compared with PCB1. PCB1 and PCB1-Bro had almost identical absorption and fluorescence spectra (Figure 2e). Under laser irradiation at 808 nm, the temperatures of PCB1 and PCB1-Bro solutions rapidly increased with irradiation time (Figure 2f), while PBS or Bro solution only exhibited a very slight temperature increase. The maximum photothermal temperatures were similar for PCB1 and PCB1-Bro (~ 52.1 °C). The photothermal conversion efficiency of PCB1 and PCB1-Bro at 808 nm was measured to be 42.8%, which was 2.5-times higher than that for PEG coated gold nanorods (17%).^[10] Additionally, the temperature increases of PCB1 and PCB1-Bro remained almost unchanged for at least 5 cycles of laser on/off (Figure S5, Supporting Information), suggesting their excellent photothermal stability.

To study photothermal effect on the activity of artificial nanoenzymes, the proteolytic activity of PCB1-Bro was investigated under laser irradiation. Two substrates were used, including the short peptide benzyloxycarbonyl-arginine-arginine-p-nitroaniline (Z-A-A-pNA) and the collagen derivative (gelatin).

Cleavage of Z-A-A-pNA led to the release of free pNA that could be colorimetrically quantified according to its absorption generated at 410 nm.^[14] Upon NIR laser irradiation for 25 min, the proteolytic activity of PCB1-Bro was enhanced by 3.5-fold as compared to that without laser irradiation (Figure 3a).

Figure 3. (a) Enzymatic activity of PCB1-Bro with or without NIR laser irradiation using the peptide (Z-A-A-pNA) as substrate. (b) GDU of PCB1-Bro with or without NIR laser irradiation using gelatin as substrate ($p < 0.001$, $n = 3$). (c) Z-stack confocal microscopy images of PCB1 or PCB1-Bro treated 3D 4T1 tumor spheroids with or without NIR laser irradiation (Scale bar = 200 μ m). (d) Quantification of fluorescence intensity of 3D 4T1 tumor spheroids at different depths. (e) Immunofluorescence collagen I staining images of 4T1 tumors after intratumoral injection of saline, PCB1 or PCB1-Bro with or without laser irradiation. Cell nucleus were stained by 4',6-diamidino-2-phenylindole (DAPI) and collagen I was stained by Alexa Fluor 488 conjugated anti-collagen I antibody. (Scale bar = 100 μ m).

The enzymatic activity of PCB1-Bro towards gelatin was expressed as gelatin digestion unit (GDU).^[15] The activity of PCB1-Bro under laser irradiation was estimated to be 1510.9 ± 27.0 GDU/g (Figure 3b), 2.3-fold higher than that without laser irradiation (663.0 ± 96.2 GDU/g). Thus, the enzymatic activity of PCB1-Bro could be triggered by photothermal heating, as the solution temperature reached to ~ 45 °C under NIR laser irradiation, which was the optimal temperature of Bro. In addition, PCB1-Bro had a good cytocompatibility even at the high

concentration of 50 $\mu\text{g/mL}$ (Figure S6, Supporting Information), permitting their biological applications.

To evaluate if the NIR light irradiation of PCB1-Bro could enhance its penetration, 4T1 multicellular spheroids were established as *in vitro* 3D tumor models and the penetration depth was measured by confocal fluorescence imaging. After incubation and NIR laser irradiation to $\sim 42^\circ\text{C}$, the fluorescence of PCB1-Bro was detected in the depth obviously deeper than that without laser irradiation (Figure 3c). At the depth of 100 μm , the fluorescence intensity of PCB1-Bro after laser irradiation was 4.4-fold of that without laser irradiation (Figure 3d). These data proved that the light irradiation enhanced the nanoparticle penetration in the 3D tumor models.

Figure 4. (a) Scheme of the treatment timeline for *in vivo* photothermic activation of collagen digestion followed by PTT treatment. (b) Real-time fluorescence images of 4T1 tumor-bearing mice after intravenous injection of PCB1 or PCB1-Bro with or without laser irradiation. (c) Quantification of fluorescence intensity of tumors as a function of post-injection time of PCB1 or PCB1-Bro. (d) IR thermal images of 4T1 tumor-bearing mice under laser irradiation for 5 min after intravenous injection of saline, PCB1 or PCB1-Bro at 6 h post-injection time. (e) Mean tumor temperature as a function of NIR laser irradiation time after intravenous injection of saline, PCB1 or PCB1-Bro at 6 h post-injection time. (f) Tumor growth curves of 4T1 tumor-bearing mice in different groups ($p < 0.05$, $n = 3$).

To validate if such enhanced nanoparticle penetration was due to the photothermic activation of collagen digestion, collagen I levels in the xenografted 4T1 tumors were assessed by immunofluorescence staining after intratumoral injection of nanoparticles with or without NIR laser irradiation (Figure 3e). PCB1 or laser treatment alone had no obvious effect on the collagen I levels, similar to the control (saline without laser irradiation). In contrast, with laser irradiation to $\sim 42^\circ\text{C}$, the green fluorescence corresponding to the collagen I was dramatically decreased by 2.6-fold as compared with the control (Figure S7, Supporting Information). This verified that PCB1-Bro had the ability to photothermally activate collagen digestion in living mice, leading to enhanced nanoparticle accumulation in tumor.

Longitudinal fluorescence imaging was performed to study if the photothermally triggered enzyme activation could enhance *in vivo* tumor accumulation of nanoparticles. After intravenous injection of PCB1 or PCB1-Bro into the tumor-bearing mice, tumors were exposed under laser irradiation. Note that the laser power was controlled to be as low as $0.2\text{ W}/\text{cm}^2$ so that the temperature was below 43°C to minimize the heat-induced apoptosis (Figure 4a). The fluorescence signals of tumors in all the groups gradually increased, and PCB1-Bro injected mice with laser irradiation had the highest tumor fluorescence intensities at each time point among all the groups (Figure 4b). At 6 h post-injection, the fluorescence intensity of tumors reached maximum for PCB1-Bro injected mice with laser irradiation, 1.42-fold higher than other groups (Figure 4c). Because PCB1 and PCB1-Bro showed similar biodistribution in the major organs (Figure S8, Supporting Information), such an enhanced accumulation of PCB1-Bro should be mainly attributed to the photothermally triggered enzymatic activation of PCB1-Bro that in-situ digested collagen in ECM and in turn improved the enhanced permeability and retention (EPR) effect.

After the enhanced accumulation of nanoparticles in tumors was achieved by photothermic activation of PCB1-Bro, the tumors were exposed under laser irradiation with a high power density of $0.3\text{ W}/\text{cm}^2$ at 6 h post-injection for PTT (Figure 4a). The tumor temperature of PCB1-Bro injected mice was higher than that for both saline and PCB1 injected mice at all the time points (Figures 4d,e). After laser irradiation for 5 min, the mean tumor temperature of PCB1-Bro injected mice was 50.5°C , 6.0 and 13.1°C higher than that of PCB1 and saline injected mice.

The PTT efficacy of PCB1-Bro was higher than that of PCB1, as PCB1-Bro completely inhibited the tumor growth for 16 days, while PCB1 failed to do so (Figure 4f). This was ascribed to the fact that only PCB1-Bro induced the mean tumor temperature (50.5°C) obviously higher than the threshold temperature (43°C) to induce cell apoptosis and necrosis.^[16] Hematoxylin and eosin (H&E) staining and immunofluorescence caspase-3 staining revealed that much more cell necrosis in the tumor tissue of PCB1-Bro injected mice relative to PCB1 injected mice with laser irradiation, while no notable necrosis was observed in other groups (Figure S9, Supporting Information).

It should be noted that the body weights of the mice after PTT showed no significant change for 16 days (Figure S10, Supporting Information). Moreover, PCB1-Bro mediated PTT did not show any damage to the major organs including heart, liver,

spleen, lung, and kidney (**Figure S11**, Supporting Information), showing the good biosafety of such treatments.

In conclusion, we have synthesized semiconducting polymer nanoenzyme (PCB1-Bro) with photothermic activation towards collagen digestion for enhanced cancer therapy. Upon NIR laser irradiation at 808 nm, the artificial nanoenzyme can convert photon energy into heat, leading to increased local temperature and thus more than 2-fold enhancement in the enzymatic activity. Thus, in situ collagen digestion can be remotely activated by NIR light after systemic administration of PCB1-Bro into living mice, resulting in the enhanced accumulation of nanoparticles in tumor. As compared with the precursor without Bro conjugation (PCB1), PCB1-Bro generates higher photothermal temperature and thus better PTT efficacy. To the best of our knowledge, this study presents the first example of an organic nanoenzyme with photothermally triggered enzymatic activity. **Although current nanoparticles are better to be used for superficial PTT owing to the limited penetration depth of 808 nm laser, it is feasible to treat interior tumors by integration with other light delivery technologies.** In addition to PTT, our approach can be used to enhance the drug delivery efficacy for other therapies such as photodynamic therapy, chemotherapy and gene therapy.

Experimental Section

See the Supporting Information for materials, instruments, synthetic procedures and supporting figures (Table S1 and Figure S1-S11).

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

Keywords: polymer nanoparticles • protein activity • cancer therapy • photothermal therapy

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Entry for the Table of Contents (Please choose one layout)

Layout 1:

COMMUNICATION

**Semiconducting Polymer
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Photothermal Activity: A semiconducting polymer nanoenzyme with photothermal activity to digest collagen is designed for enhanced cancer therapy. Upon near-infrared (NIR) light irradiation, the nanoenzyme can be activated to efficiently digest collagen in tumor extracellular matrix (ECM), leading to enhanced photothermal therapy.

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