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Tumor-microenvironment-activated *in situ* self-assembly of sequentially responsive biopolymer for targeted photodynamic therapy

Xuan Wang, Menghuan Li, Yanhua Hou, Yanan Li, Xuemei Yao, Chencheng Xue, Yang Fei, Yang Xiang, Kaiyong Cai, Yanli Zhao,* Zhong Luo*

X. Wang, M.H. Li, Y.N. Li, X.M. Yao, C.C. Xue, Y. Fei, Prof. Z. Luo
School of Life Science, Chongqing University, Chongqing 400044, China.
E-mail: luozhong918@cqu.edu.cn

M.H. Li, Prof. Y.L. Zhao
Division of Chemistry and Biological Chemistry, School of Physical and Mathematical Sciences, Nanyang Technological University, 21 Nanyang Link, 637371, Singapore.
Email: zhaoyanli@ntu.edu.sg

Y.H. Hou
Chongqing Engineering Research Centre of Pharmaceutical Sciences, Chongqing Medical and Pharmaceutical College, Chongqing 401331, China.

Y. Xiang, Prof. K.Y. Cai
Key Laboratory of Biorheological Science and Technology, Ministry of Education, College of Bioengineering, Chongqing University, Chongqing 400044, China.

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Abstract: A sequentially responsive photosensitizer-integrated biopolymer is developed for the tumor-specific photodynamic therapy, which is capable of forming long-retained aggregates *in situ* inside tumor tissues. Specifically, the photosensitizer zinc phthalocyanine (ZnPc) is conjugated with polyethylene glycol (PEG) *via* pH-labile maleic acid amide linker and then immobilized into the hyaluronic acid (HA) chain using redox-cleavable disulfide linker. The PEG segment could enhance blood circulation of the molecular carrier after intravenous administration and be shed after reaching the acidic tumor microenvironment,

allowing the remaining fragment to self-assemble into large clusters *in situ* to avoid backward diffusion and improve tumor retention. This process is driven by hydrophobic interaction and does not require additional external actuation. The aggregates are then internalized by the tumor cells *via* HA-facilitated endocytosis, and the high glutathione level in tumor cells would eventually lead to the intracellular release of ZnPc to facilitate its interaction with the subcellular lipid structures. This tumor-triggered morphology-based delivery platform is constructed with clinically tested components and could potentially be applied to other hydrophobic therapeutics.

1. Introduction

The size of nanomaterials is a crucial determining factor on their interaction pattern with the biological milieu and may fundamentally impact their functional performance in clinical environment.^[1] From a clinical standpoint, nanoparticles sized below 200 nm usually demonstrate superior tumor accumulation effect due to the enhanced permeabilization and retention (EPR) effect, and decreasing the nanoparticle size tends to enhance their tumor penetration due to better diffusive mobility.^[2] The advent of supramolecular nanotechnology provides emerging opportunities to combine the merits of both large and small nanobiomaterials, one of which is the self-assembly-enabled size switching of nanoparticles.^[3] These morphology-changeable nanoformulations could undergo specific biochemical transitions when triggered by tumor-specific stimuli and aggregate/disintegrate into certain topological nanostructures to achieve particular purposes, which may provide new translational opportunities for a wide range of tumor-related applications including imaging, drug delivery and non-conventional therapies.

Phototherapies based on nanoscale transducers have recently attracted great interest for tumor inhibition in a clinical context. Currently, there're two major classes of light-responsive nanobiomaterials according to their chemical compositions, which are the inorganic light-responsive nanostructures and organic molecules.^[4] A notable example of inorganic light-responsive nanomaterial is the black phosphorus nanosheet, which has intrinsic photodynamic, photothermal and photoacoustic capabilities as well as excellent biocompatibility and degradability in biological environment.^[5] Meanwhile, most of the light-actuable organic molecules (photosensitizers, dyes, etc) are usually hydrophobic and must be encapsulated using drug delivery nanosystems.^[6] Remarkably, morphology-changeable nanosystems have demonstrated particular relevance for of the tumor-targeted delivery of photosensitizers (PSs) for photodynamic therapy (PDT). From a practical perspective, the

therapeutic efficacy of conventional PDT agents on real-life patients has been less than satisfactory and only a handful of PSs have been approved for clinical usage against some skin cancers.^[7] A key issue that impedes the clinical implementation of conventional PDT agents is the high hydrophobicity of PSs, which are prone to rapid aggregation in aqueous environment, leading to rapid clearance from the recipient. Moreover, the aggregation of the PS molecules may also increase the risk of fluorescence resonance energy transfer and causing self-quenching.^[8] Morphology-changeable nanosystems based on self-assembly technology have shown promise to resolve these practical challenges associated with the delivery of PS molecules as they offer a non-invasive approach to stabilize the PS molecules in aqueous environment and enhance their cellular uptake. For instance, Han et al developed a chimeric peptide for the delivery of protoporphyrin IX, which could switch from spherical aggregates to a rod-like shape to accelerate the internalization by tumor cells by enhancing the interaction between the nanoaggregate and cell membrane.^[9] In another study by the same group, the authors further demonstrated that the self-assembly-enabled sphere-to-rod transition of the chimeric peptide could be exploited for the simultaneous delivery of protoporphyrin IX and tetraphenylethylene, thus enabling the aggregation-induced-emission-based apoptosis imaging of the PDT response.^[10] These emerging studies collectively demonstrated the potential clinical benefits that could be provided by these morphology-transitable nanoformulations, which pave the way for the development of more advanced PDT nanosystems with on-demand photochemical activity and low in vivo toxicity.

In this study, we report a novel acid-triggerable self-organizing supramolecular strategy for PDT with high tumor-specific accumulation/retention and optimized nano-bio interaction at subcellular level (**Scheme 1**). The proposed treatment mechanism is realized via a tumor-sensitive polymeric platform (PEG₄₀₀₀-CDM-ZnPc-S-S-HA) that comprises only clinically-tested biocompatible components.^[11] This multi-segment polymeric platform is hierarchically constructed with three basic components. Specifically, zinc phthalocyanine (ZnPc) is first conjugated with polyethylene glycol (PEG₄₀₀₀) chains via acid-sensitive maleic acid amide linker (-CDM-) and then immobilized onto the hyaluronic acid (HA) backbone using redox-cleavable disulfide linker (-S-S-).^[12] Thanks to the high hydrophilicity and low immunogenicity of the HA and PEG segments as well as its optimized hydrodynamic size, this nanoplatform could cross the erratically aligned endothelial cells in the tumor vasculature and effectively accumulate in the tumor tissues via passive targeting and penetrate deep into the tumor tissues. Afterwards, the PEG segment could be readily removed by the acid-triggered cleavage of the CDM linkers and the remaining components would self-assemble

into nanoparticles due to the changes in the hydrophobicity/hydrophilicity balance of the biopolymers. Considering that the PEG shedding rate is positively correlated to the environmental acidity and that the tumor core is more acidic than the edge regime, the HA-based biopolymer could gradually spread into the whole tumor and form non-refluxable water-soluble aggregates in a self-regulated manner, leading to a more homogenous drug deposition pattern throughout the tumor stroma as well as enhanced tumor retention. It was anticipated that this aggregation-enabled sequential tumor targeting could enhance both the efficiency and specificity of PS delivery to the tumor tissues. Eventually, the ZnPc molecules could be detached from the HA backbone under the stimulation of GSH and form small aggregates in the cytoplasm, which may enhance the interaction between the lipophilic PS and the lipid membrane structures in the intracellular environment,^[13] thus further amplifying the cytotoxic damage of ZnPc-generated reactive oxygen species (ROS) under light irritation.

2. Results and Discussion

2.1 Characterizations on the biopolymer

The chemical structure of the as-prepared PEG₄₀₀₀-CDM-ZnPc-S-S-HA was evidenced by the NMR spectrum (**Figures S1-S3**) and UV-vis spectrum (**Figure 1d**). Specifically, the PEG₄₀₀₀-CDM-ZnPc-S-S-HA molecules have a broadened absorption peak at 720 nm due to the face-to-face molecular stacking thereof,^[14] while the minor peak at 660 nm apparently red shifted with reduced absorption intensity due to the π - π interactions.^[15] The loading ratio of ZnPc on the HA backbone was around 8% according to the UV absorption data (**Figure S4**). Thanks to the high loading ratio of ZnPc in the biopolymer-based nanoplatform, the ROS quantum yield of PEG₄₀₀₀-CDM-ZnPc-S-S-HA under 720 nm laser was found to be around 0.4 (**Figure S5**), which is comparable to other ZnPc-incorporated PDT nanosystems.^[16] We then investigated the aggregation of the HA-based polymeric PDT platform (**Figure 1a**) in aqueous solution after acid incubation using transmission electron microscopy (TEM). After the treatment with weak acid (pH 6.5), the width of these characteristic peaks slightly decreased due to the acid-induced cleavage of the CDM bond and the recovery (**Figure 1b,c**), and the visualized results evidently confirmed that the acid-induced PEG-removal from the ZnPc moiety caused the remaining ZnPc-S-S-HA molecules to self-assemble into roughly spherical clusters with an average size of around 200 nm. Meanwhile, we also carried out the gel permeation chromatography (GPC) analysis (**Figure S6** and **Table S1**) for the biopolymer sample series. The results indicate that the average molecular weight of PEG₄₀₀₀-CDM-ZnPc-S-S-HA decreased by around 8000 after the incubation in acidic buffer (pH 6.5), which was evidently

attributed to the cleavage of the CDM linker and the resultant removal of the PEG segment. The morphological impact caused by the PEG shedding was further analyzed by dynamic light scattering (Figure 1e), which revealed that the PEG₄₀₀₀-CDM-ZnPc-S-S-HA was originally in a monodispersed state in phosphate-buffered saline (PBS) solutions of neutral pH (hydrodynamic size: 18 nm), but could readily form aggregates sized around 200 nm in acidic PBS (pH 6.5). The PEG shedding-induced size change was also in good accordance with the zeta potential results, which revealed that the negativity of the zeta-potential for the PEG₄₀₀₀-CDM-ZnPc-S-S-HA reduced slightly (**Figure S7**) after the incubation in acidic buffer. Subsequently, the PEG-removal process was kinetically investigated by fluorescence imaging and found to be very time-efficient (**Figure S8**), as results showed that all PEG segments were removed after incubation at pH 6.5 for 6 hours. According to the fluorescent studies with I330 and I339, the critical micelle concentration of biopolymer after PEG removal (ZnPc-S-S-HA) was 1.5 $\mu\text{g/mL}$ (**Figure S9**). As suggested by previous insights on tumor vasculature, nanoparticles in the size range of 50-200 nm could move freely across the abnormal tumor blood vessels through the fenestrations between those poorly aligned endothelial cells.^[17] Consequently, it's theorized that the in-situ formation of large size ZnPc-S-S-HA aggregates (>200 nm) in the tumor microenvironment could prevent the therapeutic agents from leaking back to the blood so as to prolong their tumor retention.

2.2 GSH-triggered ZnPc release in solution

A common feature of various tumor cells is the upregulated expression of bioreductants (e.g. GSH) in the intracellular environment to compensate the elevated oxidative stress,^[18] which has been exploited in this study as a regulatory stimulus for the controlled release of ZnPc from the PEG₄₀₀₀-CDM-ZnPc-S-S-HA aggregate. As shown in Figures 1f and **S10**, no apparent ZnPc release was observed under both pH 6.5 and 7.4, demonstrating the excellent ZnPc encapsulation efficiency of PEG₄₀₀₀-CDM-ZnPc-S-S-HA regardless of the aggregation state. In comparison, a burst release profile was detected when GSH (10 mM) was added into the PBS for incubation, under which more than 60% of the loaded ZnPc was released in the initial 6 hours and the total ZnPc release ratio after 24 hours was as high as 80%. We also found that the loaded ZnPc could be rapidly released from the biopolymer under pH 7.4+10 mM GSH, but the release rate is slightly slower than that under pH 6.5+10 mM GSH, which could be explained by the steric hindrance of PEG segments. Moreover, we observed that the ZnPc release rate was positively correlated to the GSH concentration in the incubation solution and only negligible ZnPc leakage was detected at the GSH concentration of 0.01 mM that corresponds to the GSH level in the intercellular environment,^[12a,19] further validating the

GSH-regulated ZnPc release mechanism (**Figure S10**). The GSH-triggered alteration in ZnPc release patterns immediately suggests the redox-responsiveness of the macromolecular system, which is vital for improving the therapeutic index of the photodynamic treatment.

2.3 Cellular uptake of the biopolymer

As for the *in vitro* evaluation of the biopolymer formulation, we first examined the cellular uptake efficiency of fluorescein isothiocyanate (FITC) labeled samples under neutral pH (7.4) using confocal laser microscopy (CLSM) (**Figures 2a-c and S12-S15**). As shown in Figure 2a-c and **S12**, 4T1 cells incubated with PEG₄₀₀₀-CDM-ZnPc@FITC-S-S-HA showed only minimal FITC fluorescence after incubating for 24 hours, of which the relative rate of uptake was only 20%. In comparison, the total FITC fluorescence in those treated with ZnPc-S-S-HA was evidently stronger, which has a relative uptake rate of around 60% (**Figures 2c and S16**). The drastic discrepancy in the cellular uptake efficiency of the two samples was apparently caused by the PEG segments that could impede the binding between HA and the CD44 receptors on tumor cell surface. It should also be mentioned that the FITC fluorescence in the 4T1 cells dropped significantly when the tumor cells were pre-incubated with free HA to saturate the CD44 on the cytoplasm membranes, which could be explained by the blockade of the CD44-mediated endocytic pathway. The CLSM observations were also supported by the quantitative results of flow cytometric analysis showed in **Figures 2d and S13-S15**, in which the mean FITC fluorescence in the ZnPc@FITC-S-S-HA group was around 3 times as high as that in the PEG₄₀₀₀-CDM-ZnPc@FITC-S-S-HA group after 24 h of incubation. Moreover, we also monitored the uptake efficiency of ZnPc@FITC-S-S-HA by MCF10 cells using a similar experimental set-up (**Figure 2e,e1,f,f1,g**), which is a normal cell line with low expression level of CD44 (**Figure 2h**). The CLSM and flow cytometry results collectively demonstrated that the uptake amount of ZnPc@FITC-S-S-HA by MCF-10 cells remained at a relatively low level after 4 h of incubation under both HA-pretreated and non-pretreated conditions. Comparative analysis further revealed that the amount of ZnPc@FITC-S-S-HA uptake by HA-pretreated 4T1 cells and CD44-negative MCF10 cells was at a similarly low level, again validating the HA-enabled tumor specificity. The observation that the cellular uptake efficiency of the biopolymer was highly dependent on CD44 availability evidently suggested the CD44-targeting capability of HA and the resultant facilitative effect on the cellular uptake of the HA-based biopolymer by CD44-overexpressed tumor cells.

In addition to the cellular uptake evaluation under neutral pH using pre-synthesized samples, we also investigated the potential variations in the cellular uptake of PEG₄₀₀₀-CDM-ZnPc@FITC-S-S-HA under different pH conditions. Consistently, it could be observed in

Figure S17 that the amount of PEG₄₀₀₀-CDM-ZnPc@FITC-S-S-HA uptake by 4T1 cells under pH 7.4 was at a negligibly low level, as revealed by the sparse FITC fluorescence in panel b3. In comparison, abundant FITC fluorescence was observed in 4T1 cells incubated with PEG₄₀₀₀-CDM-ZnPc@FITC-S-S-HA under pH 6.5. The enhanced cellular uptake of PEG₄₀₀₀-CDM-ZnPc@FITC-S-S-HA at pH 6.5 was attributed to the acid-cleavability of the CDM linker and the subsequent PEG removal under tumor-microenvironment-mimetic conditions, which evidently suggested that the PEG₄₀₀₀-CDM-ZnPc-S-S-HA formulation could significantly improve the delivery efficiency of ZnPc to target tumor cells via acid-triggered PEG shedding and self-assembly based aggregation.

2.4 ROS generating capability of the biopolymer

One of the key performance indicators for the evaluation of PDT efficacy is the intracellular ROS generation rate, which was studied here via dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay. DCFH-DA is non-fluorescent molecule but could be oxidized by the intracellular ROS into 2',7'-dichlorofluorescein (DCF), which has strong fluorescence at the wavelength of around 529 nm and therefore could be exploited for the in-situ tracking of the ROS generated by the PDT treatment. As shown in **Figure 4a**, strongest DCF fluorescence (green) was detected in the intracellular compartment of ZnPc-S-S-HA/NIR treated 4T1 cells, while the DCF fluorescence in the ZnPc-HA/NIR, PEG₄₀₀₀-CDM-ZnPc-S-S-HA/NIR, PEG₄₀₀₀-CDM-ZnPc-HA/NIR groups was as low as the control group. Particularly, the mean DCF fluorescence intensity in the ZnPc-S-S-HA/NIR group increased from 17.2 to 137 after treatment, while the fluorescence increase in the PEG₄₀₀₀-CDM-ZnPc-HA/NIR was almost negligible (Figure 4b). The comparative analysis of the ROS generation efficiency in the four groups above evidently suggests the enhancing effect of the sequential pH/redox dual-responsive ZnPc delivery strategy. The distinct ROS production patterns in different samples were further investigated quantitatively using flow cytometry and the results are highly consistent (Figure 4c). Additionally, we also studied the ROS generating efficiency of PEG₄₀₀₀-CDM-ZnPc-S-S-HA under different pH conditions using DCF-DA assay kit, of which the results could be used as an indicator of their PDT effectiveness in clinically relevant conditions. As demonstrated by **Figure S18**, the DCF fluorescence was much stronger in 4T1 cells incubated under pH 6.5 than those at pH 7.4, which was in good accordance with the superior uptake efficiency of PEG₄₀₀₀-CDM-ZnPc-S-S-HA under acidic pH due to the PEG-removal-induced aggregation.

2.5 Tumor spheroid assay on the penetration ability of the biopolymer

To further investigate the penetration enhancement by the size-switching mechanism, we have established the 4T1 tumor spheroid models and incubated them with various FITC-labeled biopolymer samples. As shown by the fluorescence imaging results in Figure 3 and quantitative analysis in **Figure S19**, the green fluorescence in the PEG₄₀₀₀-CDM-ZnPc-S-S-HA + lactic acid supplement (pH 6.5) group has penetrated deep into the tumor spheroids and distributed homogeneously throughout the spheroid models. We also observed that the ZnPc-S-S-HA could enter the tumor spheroid at reduced efficiency (approximately 30% less than the PEG₄₀₀₀-CDM-ZnPc-S-S-HA + lactic acid group), but the distribution pattern was much less homogeneous. Meanwhile, the tumor penetration of PEG₄₀₀₀-CDM-ZnPc-S-S-HA was visibly lower, for which the majority of the FITC fluorescence still remained at the edge of the tumor spheroids. Moreover, it was also demonstrated the HA-pretreated tumor spheroids showed the least biopolymer uptake, which could be explained by impeded endocytosis due to the HA-saturated CD44 glycoproteins on the tumor cell membrane. The changes in the tumor penetration efficiency of different biopolymer samples evidently support the proposed tumor-microenvironment-responsive size-switching mechanism and its facilitative effect on the tumor-targeted delivery of photosensitizers.

2.6 Evaluation on the PDT efficacy in vitro

Based on the promising ROS generation efficiency of the PEG-shedding polymeric agents on 4T1 cells, we further investigated the in vitro PDT efficacy of the polymeric nanocarrier using multiple complementary techniques. According to the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (**Figure S20**), the HA-based substrate itself was highly biocompatible, as indicated by the high survival rate of 4T1 cells in those non-NIR-treated groups (PEG₄₀₀₀-CDM-ZnPc-S-S-HA, ZnPc-S-S-HA, PEG₄₀₀₀-CDM-ZnPc-HA, ZnPc-HA and PEG₄₀₀₀-CDM-ZnPc-S-S-HA at pH 6.5). However, significant tumor cell growth inhibition (survival rate: 15%) was observed when ZnPc-S-S-HA was used in conjunction with external NIR illumination (720 nm, 10 min), while the inhibition effect in the PEG₄₀₀₀-CDM-ZnPc-S-S-HA/NIR group was only at a modest level of around 79%. The PEG₄₀₀₀-CDM-ZnPc-S-S-HA/NIR + lactic acid (pH 6.5) group also demonstrated pronounced tumor inhibition effect (survival rate: around 14%) similar to ZnPc-S-S-HA, indicating the effective PEG shedding in acidic conditions. It is also worth mentioning that ZnPc-S-S-HA has demonstrated evidently higher tumor inhibition effect than non-disulfide-containing ZnPc-HA group under NIR illumination, which is suggestive of the therapeutic contribution of the redox-triggered ZnPc release mechanism. The results above consistently supported our hypotheses that the self-organized aggregation of the polymeric platform after acid-induced

PEG-shedding and the subsequent GSH-triggered ZnPc release in the tumor intracellular compartment are beneficial for enhancing the PDT efficacy.

The treatment-induced cellular damaging patterns were further analyzed with the cell live/dead staining assay and flow cytometry. As shown in **Figure 5a**, we did not detect any dead cell in the biopolymer-treated groups when no NIR laser was applied, indicating the biocompatibility of the samples. The largest amount of shrunken and rounded dead cells appeared in the ZnPc-S-S-HA/NIR group, which immediately suggested the potent tumor cell damage induced by the PDT-generated ROS. In comparison, only small number of scattered dead cells was found in the other groups. Flow cytometric apoptosis analysis consistently revealed that the tumor cell apoptosis was most severe in the ZnPc-S-S-HA/NIR group, which has an apoptotic ratio of 61.4% compared to the 11.4% of the PEG₄₀₀₀-CDM-ZnPc-HA/NIR group (Figure 5b, d). The difference in the pro-apoptotic capability between the PEG₄₀₀₀-CDM-ZnPc-HA/NIR and ZnPc-S-S-HA/NIR group was also supported by the results of live/death imaging of 4T1 cells incubated with PEG₄₀₀₀-CDM-ZnPc-HA/NIR under different pH conditions (**Figure S21**).^[15] Remarkably, it could be observed that the death rate of cells treated by PEG₄₀₀₀-CDM-ZnPc-HA/NIR at pH 6.5 was at the same level to that of the ZnPc-S-S-HA/NIR group under neutral pH, while most of the 4T1 cells survived the treatment with PEG₄₀₀₀-CDM-ZnPc-HA/NIR when the pH value was 7.4. The cell live/death imaging and flow cytometry results collectively confirmed therapeutic benefits of the acid-triggered aggregation and redox-triggered ZnPc release in vitro. The apoptosis-inducing effect of the treatment-induced PDT was also mechanistically confirmed by western blot assays, where the expression level of pro-apoptotic BAX proteins in the ZnPc-S-S-HA + NIR group increased by 250% in comparison with the control group, while the expression level of anti-apoptotic Bcl-2 proteins decreased by 20% (Figure 4c and **S22**). The drastic changes in the relative expression levels of BAX and Bcl-2 confirmed that the biopolymer-mediated PDT could activate the Capase-3 apoptosis pathways and exert potent cytotoxicity against the tumor cells.

2.7 Evaluation of the PDT efficacy in vivo

The PDT efficacy of the polymeric platform was further investigated in vivo on 4T1-tumor-bearing mouse models. We firstly measured the circulation stability of the biopolymers via Cy5 staining and found that the elimination half-life of PEG₄₀₀₀-CDM-ZnPc-S-S-HA was as long as 8 hours, which is much longer than the 5 h of the ZnPc-S-S-HA samples (**Figure S23**). The in vivo distribution of various polymeric samples after systemic administration was then monitored via fluorescence imaging. By comparing the fluorescence patterns in Figure 6a, it

was observed that most of the PEG₄₀₀₀-ZnPc-S-S-HA accumulated in the kidneys and liver of the mouse models 24 h post intravenous injection due to the uptake by the mononuclear phagocyte system, while only a modest proportion of the administered drug were deposited in the tumor tissues. In comparison, the Cy5 fluorescence in mice administered with PEG₄₀₀₀-CDM-ZnPc-S-S-HA was mostly enriched at the tumor region (**Figure 6a,b**), indicating the significantly reduced uptake of Cy5-conjugated polymers by other non-specific tissues or organs. Quantitative fluorescence evaluation of the extracted tumor tissues and key organs of the mice demonstrated that the relative tumor accumulation of Cy5 increased from 18% in the PEG₄₀₀₀-ZnPc-S-S-HA group (Figure 6c, d) to 43% in the PEG₄₀₀₀-CDM-ZnPc-S-S-HA group (Figure 6d). The significant increase of tumor-specific Cy5 accumulation was ascribed to the acid-triggered self-organizing aggregation of the ZnPc-S-S-HA after PEG shedding as well as the targeting effect of the HA content. Furthermore, we investigated the multi-responsive ability of the nanoplatform in vivo via heterogenous fluorescence labeling of PEG₄₀₀₀-CDM-ZnPc-S-S-HA. Typically, the PEG segment was labeled with FITC (green fluorescence), while the ZnPc molecules were labeled with Cy5 (red fluorescence). As shown by the fluorescence imaging results of the tumor tissue slices (**Figure S24**), the green and red fluorescence were discretely distributed in the tumor tissues with minimal overlapping. Moreover, most of the green fluorescence remained in the intercellular regions, while the red fluorescence was found within the tumor cells and located near the cell nuclei, which is in good accordance with the sequential pH/GSH responsiveness of the biopolymers and may explain the variation between the antitumor efficacy of ZnPc-S-S-HA and ZnPc-HA in the cellular experiments.

Taking advantage of the therapeutically favorable biodistribution profile and tumor-responsive ZnPc release, mice with the combined treatment of PEG₄₀₀₀-CDM-ZnPc-S-S-HA and NIR showed the most effective tumor inhibition (**Figures 7a** and **S25**), in which the average tumor volume and weight reduced by 1.93 cm³ and 1.6 g compared to the blank control, respectively. It was also noticed that only the PEG₄₀₀₀-CDM-ZnPc-S-S-HA/NIR group showed declining trends in tumor volumes (Figure 7b). As a result of the enhanced tumor inhibition effect, mice in the PEG₄₀₀₀-CDM-ZnPc-S-S-HA/NIR group also displayed prolonged survival than the other groups (Figure 7c), which displayed a median survival time of 65 days. Mice in the other groups were all deceased before day 50 due to tumor burden. The superior PDT efficacy conveyed by PEG₄₀₀₀-CDM-ZnPc-S-S-HA was also supported by the histological analysis via terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), which presented the highest degree of apoptosis among all treatments (Figure 6d).

In addition to the efficacy evaluations, we also investigated the *in vivo* safety of the polymeric platform via weight monitoring (**Figures S26 and S27**) and histopathological examination. The average mouse body weight in all groups remained at a comparable level throughout the treatment period, indicating the excellent biocompatibility of the macromolecular system and the safety of the PDT treatment. Meanwhile, no apparent cellular and tissue damage was observed in histological analysis of the hematoxylin and eosin (H&E) stained organs/tumor tissues (**Figure S28**). The hemocompatibility of the biopolymer samples was also studied by investigating the changes in vital blood indexes including red blood cell indexes, blood platelet counts, white blood cell indexes, mean corpuscular hemoglobin levels, hemoglobin levels, hematocrit values and mean corpuscular volumes, and the results collectively demonstrated that all the blood parameters remained within normal range after the biopolymer-mediated PDT (**Figure S29**).^[20] Additionally, we have monitored the temperature changes in the mouse models after the NIR laser treatment (**Figure S30**) using a thermographic camera and found only slight temperature increase in the laser-treated tumor region, while the systemic temperature of the mouse models remained normal, indicating that the ZnPc-incorporated biopolymer didn't have apparent photothermal effect under NIR irradiation and minimize the risk of collateral thermal damage. These observations evidently suggest that the PEG₄₀₀₀-CDM-ZnPc-S-S-HA-mediated PDT could be safely used for tumor treatment, which further added to its clinical translation potential.

3. Conclusions

In summary, we have successfully developed a biodegradable and acid/redox sequentially responsive polymeric platform for tumor-targeted PDT. This molecular carrier could stably circulate in body and self-assemble into large size clusters upon entering the tumor microenvironment by shedding the PEG segment in a self-regulated manner, which would enhance the tumor-specific retention of PS agent. After the HA-facilitated endocytic uptake by the tumor cells, the high level of intracellular GSH could further cleave the disulfide bond between ZnPc and the HA backbone, thus facilitating the interaction between ZnPc and subcellular lipid structures to amplify the cytotoxic damage. These features collectively contributed to the potent photodynamic activity against tumor cells observed from the *in vitro* and *in vivo* experiments, achieving pronounced tumor inhibition effects and prolonged mouse survival. Furthermore, the reported delivery strategy could also be readily applied for the tumor-targeted delivery of other hydrophobic therapeutic agents, making it a versatile molecular platform for the treatment of a variety of solid tumors.

4. Experimental Section

Synthesis of ZnPc. 4-Nitrophthalimide (4.1 g), urea (10 g) and ammonium molybdate (0.05 g) were mixed in a three-necked flask and heated until melting. Zinc chloride hexahydrate (1.2 g) was then added and the mixture was continuously heated at 160 °C until no more bubbles appeared. Samples were extracted after cooling and washed first with HCL (1 mol) and NaOH solution (1 mol) and then ultrapure water, and anhydrated under vacuum to obtain the blue-violet product. Then, the product obtained above and sodium sulfide nonahydrate (2.88 g) were mixed with DMF (15 mL) and heated to 60 °C in a three-necked flask under intense stirring. After 1 hour, the mixture was washed with water and dried under vacuum to obtain solid substance with dark green color (ZnPc with 4 residue amine groups).

Synthesis of 2,2'-dipyridyldisulfide. Disulfide dichloride (3.75 g) was dissolved in ethanol (10 mL) and then added with acetic acid (0.4 mL). Subsequently, 3-mercaptopoacetic acid (0.9 g) was dissolved in ethanol and added dropwise to the above mixture, the reaction was carried at room temperature for 20 hours. The solution was dried under vacuum and the product was purified by passing through an alumina column with the mixed solution of dichloromethane, ethanol and acetic acid (volume ratio: 3: 2: 0.2). The white substances are collected and dried under vacuum to obtain 2,2'-dipyridyldisulfide.

Synthesis of HA-HS. HA (200 mg) was added to hexamethylenetetramine buffer at pH 5, and then stirred with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 500 mg) and *N*-hydroxysuccinimide (NHS, 300 mg) for 3 h to activate the carboxyl groups. Thiol ammonia hydrochloride (300 mg) was then added and the reaction was carried out under a nitrogen atmosphere for 24 hours. The crude product was dialyzed at the molecular weight cut-off (MWCO) of 1000 for 2 days, and then precipitated by ethanol. The purified product was denoted as HA-HS.

Synthesis of PEG₄₀₀₀-CDM. CDM (0.276g) is dissolved in anhydrous dichloromethane and added with oxaloyl chloride (0.378g) and DMF (40 μ L) as catalyst. The reaction was first performed under ice/water bath for 30min and then to room temperature for 6h to obtain acylated CDM. After vacuum drying, polyethylene glycol monomethyl ether 4000 (800mg) was added for reaction in anhydrous dichloromethane, and pyridine (30 μ L) was added as catalyst, After the reaction at 25 °C for 6 hours, saturated ammonium chloride solution was added to extract the organic phase, which was collected and dried in vacuum. Then the organic phase was dissolved in dichloromethane and precipitated twice anhydrous ether. The resulting product was dried under vacuum to obtain the white powder (PEG₄₀₀₀-CDM).

Synthesis of PEG₄₀₀₀-CDM-ZnPc. PEG₄₀₀₀-CDM (70mg) and ZnPc (48mg) were added to 30ml of anhydrous tetrahydrofuran at the stirring speed of 400 rpm, and the reaction temperature was 30 °C, the reaction would last 24h under nitrogen protection. The intermediate product was dried in vacuum and then dissolved in dichloromethane for 5min of ultrasound treatment and the supernatant was collected by centrifugation. The purification was repeated 3 times until no green color was found in the supernatant. The dichloromethane in the supernatant was removed by low-temperature vacuum drying, and the dark green solid (PEF4000-CDM-ZnPc) was obtained.

Synthesis of PEG₄₀₀₀-CDM-ZnPc-S-S-pyridine. Carboxyl disulfide (12 mg) was firstly dissolved in 10ml DMF and then added with NHS (25 mg) and EDC (20 mg). The incubation would last 30min at room temperature to activate the carboxyl group was activated. CDM-PEG₄₀₀₀-ZnPc (200 mg) was then added under nitrogen protection and incubated at room temperature for 24 hours, the stirring speed was set at 400 rpm. After the reaction, the DMF content was removed by vacuum drying. The product was dissolved in water and dialyzed at the MWCO of 1000 for 3 days to remove excessive EDC, NHS and residual carboxyl disulfide pyridine. The purified samples were anhydrated to obtain a dark green solid (PEG₄₀₀₀-CDM-ZnPc-S-S-pyridine).

Synthesis of PEG₄₀₀₀-CDM-ZnPc-S-S-HA. The synthesized HA-HS (38 mg) was dissolved in PBS (10 mL), while PEG₄₀₀₀-CDM-ZnPc-S-S-pyridine (200 mg) is dissolved in DMF (10 mL). The two samples were then mixed and reacted under the protection of nitrogen at room temperature for 48 hours, at the stirring speed of 450 rpm. The product was dialyzed at the MWCO of 3000 for 3 days to remove unreacted agents and then precipitated with ethanol. The precipitates were extracted and moisture was removed by vacuum freeze-drying to get a dark green solid (PEG₄₀₀₀-CDM-ZnPc-S-S-HA)

Synthesis of ZnPc-S-S-HA. PEG₄₀₀₀-CDM-ZnPc-S-S-HA. was treated at pH 6.5 for 6 h and then precipitated with ethanol to obtain ZnPc-S-S-HA, during which the PEG segments were removed by cleaving the CDM linker.

Synthesis of PEG₄₀₀₀-ZnPc-S-S-HA. To start with, succinic anhydride (30 mg), DMAP (4 mg), triethylamine (31 mg), CH₃-PEG₄₀₀₀-OH (800 mg) was mixed in dichloromethane for 24 h for reaction, and the raw product was extracted twice with dichloromethane. The organic phase was collected and dried in vacuo to give the product CH₃-PEG₄₀₀₀-COOH. CH₃-PEG₄₀₀₀-COOH (400 mg), EDC (57 mg) and NHS (33 mg) were dissolved in 50ml of DMF and stirred for 30 min to activate the carboxyl groups. ZnPc (120 mg) was then added under nitrogen protection and reacted for another 24 hours. The raw product was collected by

vacuum drying and then re-dissolved in dichloromethane. The solution was centrifuged to collect the supernatant solution so as to remove excessive the ZnPc. The product PEG₄₀₀₀-ZnPc was collected by vacuum drying. Subsequently, 2,2'-dipyridyldisulfide (12 mg) was dissolved in DMF (10 mL) and added with NHS (25 mg) and EDC (20 mg). The mixture was stirred for 30 min to activate the carboxyl groups. Afterwards the as-synthesized PEG₄₀₀₀-ZnPc (200 mg) was added under the protection of nitrogen. The reaction would be carried out at room temperature for 24 h at the stirring speed of 400 rpm. Subsequently, the sample was treated with vacuum drying to remove the DMF solvent. The product was dissolved in water and dialyzed at the MWCO of 1000 for 3 days to remove unreacted agents, and then freeze-dried under vacuum to afford the dark green solid PEG₄₀₀₀-ZnPc-S-S. Finally, HA-HS (38 mg) in PBS (10 mL) and PEG₄₀₀₀-ZnPc-S-S (190 mg) in DMF (10 mL) were mixed under nitrogen protection under 25 °C and stirred for 48 hours. When the reaction was complete the solution was dialyzed at the MWCO of 3000 for 4 days, and eventually freeze-dried to give the PEG₄₀₀₀-ZnPc-S-S-HA.

FITC labeling of PEG₄₀₀₀-CDM-ZnPc-S-S-HA for the analysis of PEG shedding. To investigate the acid-triggered PEG-detachment feature of the PEG₄₀₀₀-CDM-ZnPc-S-S-HA molecules, FITC was conjugated to the PEG segment for fluorescent labeling. Specifically, FITC (10.5mg) and propargylamine (20 mg) were dissolved into 18mL of pure water and reacted for 24 hours at room temperature (25 °C), and the sample was treated with vacuum drying to remove the solvent. Acetone was added into the remaining solids to remove unreacted propargylamine and thus obtain the FITC-propargylamine. Then, propargyl-FITC (44.5mg) and N₃-PEG₄₀₀₀-OH (400mg) were dissolved into pure water (25mL), and then added with CuSO₄ (5mg) and ascorbic acid (5mg) under nitrogen protection to trigger the click reaction, the reaction would last 24h to obtain FITC-PEG₄₀₀₀-OH. FITC-PEG₄₀₀₀-OH could react with acylated CDM in anhydrous dichloromethane with pyridine (30 uL) as catalyst. After reacting at 25 °C for 6 hours, saturated ammonium chloride solution was added to extract the organic phase, which was collected and dried in vacuum to obtain FITC-PEG₄₀₀₀-CDM. Finally, the FITC-PEG₄₀₀₀-CDM-ZnPc-S-S-HA was synthesized according to the above steps for PEG shedding test.

FITC labeling of PEG₄₀₀₀-CDM-ZnPc-S-S-HA for endocytosis evaluation. For the fluorescent tracking of the HA-based biopolymer in vitro, the ZnPc moieties of the PEG₄₀₀₀-CDM-ZnPc-S-S-HA samples were conjugated with FITC. Specifically, PEG₄₀₀₀-CDM-ZnPc-S-S-HA (50 mg) was dissolved in PBS solution (25 mL) and added with FITC (5 mg). The reaction temperature was maintained at room temperature (25 °C) and the stirring speed was

300 rpm. The underlying rationale is that the NHS-ester of FITC could react with the amino groups of the ZnPc moieties and forming an isothiourea bond for stable labeling. The reaction temperature was maintained at room temperature (25 °C) and the stirring speed was 300 rpm. Afterwards the solution was dialyzed at the MWCO of 1000 for 3 days to remove residue FITC, affording the PEG₄₀₀₀-CDM-ZnPc@FITC-S-S-HA product for endocytosis analysis.

Synthesis of dual-labeled FITC-PEG₄₀₀₀-CDM-ZnPc@Cy5-S-S-HA. FITC-PEG₄₀₀₀-CDM-ZnPc-S-S-HA (10 mg) was dissolved in PBS solution (15 mL) and added with Cy5 (3mg). The reaction temperature was maintained at room temperature (25 °C) and the stirring speed was 300 rpm. Afterwards the solution was dialyzed at the MWCO of 1000 for 3 days to remove residue Cy5 to obtain the FITC-PEG₄₀₀₀-CDM-ZnPc@Cy5-S-S-HA product for fluorescent imaging of mouse tumor slices.

Determination of the ROS quantum yield. The ROS quantum yield of the ZnPc-incorporated biopolymer was determined via Methylene Blue (MB) assay.^[21] UV spectrophotometer was employed to investigated the singlet oxygen (¹O₂) levels and 1,3-diphenylisobenzofuran (DPBF) was used as the probe, which has an absorption wavelength of 408 nm. The cuvettes containing the biopolymer samples were placed in a dark room and irradiated for 0, 60, 120, 180, 240 and 360 seconds by a 720 nm laser, while the MB sample was treated with monochromatic light.

The ROS quantum yield of ZnPc-S-S-HA was calculated based on the following equation:

$$\Phi_{NP} = \Phi_{MB} (K_{NP} I_{MB}) / (K_{MB} I_{NP})$$

where Φ_{NP} and Φ_{MB} were the ROS quantum yield for ZnPc-S-S-HA and MB respectively. K_{NP} and K_{RB} represent the slope of plot $\ln(A_0/A)$ versus illumination time of ZnPc-S-S-HA and MB, respectively. I_{NP} and I_{MB} were calculated from the areas of their absorption spectra from 400-800 nm, respectively.

GSH-triggered ZnPc release profile. PEG₄₀₀₀-CDM-ZnPc-S-S-HA (2mg) was dissolved in 1 mL of pure water and then incubated at different conditions including pH 7.4, pH 6.5, and pH6.5 + GSH (10mM). All samples were kept under dark environment on a mechanical shaker. The released ZnPc was collected via low-speed centrifugation (1000 rpm, 5min) and re-dissolved with DMF for spectroscopic analysis. The light absorption of the samples at 500-900 nm was measured at different time points of 30 min, 1 hour, 2 hours, 3 hours, 4 hours, 6 hours, 12 hours and 24 hours.

Drug release profile under GSH stimulation. To monitor the ZnPc release kinetics under different GSH concentrations, PEG₄₀₀₀-CDM-ZnPc-S-S-HA was added to PBS (pH = 6.5) at a

concentration of 0.2 $\mu\text{g/mL}$ and then treated by 10 mM, 1 mM and 0.01 mM of GSH, respectively. The ZnPc absorption was detected by fluorescence spectrophotometer after 30 min, 1 hour, 2 hours, 3 hours, 4 hours, 6 hours, 12 hours and 24 hours of incubation.

Acid-triggered PEG removal. In order to study the PEG detaching kinetics of PEG₄₀₀₀-CDM-ZnPc-S-S-HA in tumor microenvironment, FITC-PEG₄₀₀₀-CDM-ZnPc-S-S-HA was stored in a dialysis bag of MWCO 4000 and incubated at pH 6.5. An aliquot was extracted at different time points of 0.5, 1, 1.5, 2, 4, 6, 8, 10 and 24 hours and the FITC fluorescence intensity was measured to indicate the relative amount of PEG detachment.

UV-vis absorption spectrum. The as-synthesized ZnPc, PEG₄₀₀₀-CDM-ZnPc-S-S-HA and acid-treated PEG₄₀₀₀-CDM-ZnPc-S-S-HA were solubilized at equivalent ZnPc concentration and the corresponding UV absorption in the range of 500-900 nm was measured on a UV spectrometer. ZnPc was solubilized in DMF, PEG₄₀₀₀-CDM-ZnPc-S-S-HA and acid-treated PEG₄₀₀₀-CDM-ZnPc-S-S-HA were solubilized in water.

Measurement of the critical micelle concentration. The self-assembly of the biopolymer was studied using pyrene as the probe. Firstly, a series of aqueous acetone solution (6×10^{-7} M) were prepared and stored in centrifuging tubes, and 1 mL solution was taken from each tube. The extracted solution was allowed to stand overnight, and then the biopolymer solution at different concentrations ($10^{-5} \sim 1$ mg/mL) were added to the centrifuge tube, and final Pyrene concentration was maintained at 6×10^{-7} M. The solution was shaken overnight under dark. The excitation spectrum was analyzed by a fluorescence spectrophotometer (LS50B, PerkinElmer, USA) at the emission wavelength of 390 nm. Finally, the relationship between the intensity ratio of I_{339}/I_{333} and the logarithm of polymer concentration as well as the fluorescence absorption spectra under different concentrations were plotted using the obtained data.

Establishing the standard curve for the calculation of the ZnPc loading. To investigate the loading ratio of ZnPc onto the HA backbone, we first measured the concentration of ZnPc to establish the standard curve to determine the concentration of PEG₄₀₀₀-CDM-ZnPc-S-S-HA. ZnPc was detached under pH 6.5 and GSH treatment, and collected by centrifugation, and eventually the UV absorption was measured on a UV-vis spectrometer.

GPC analysis on molecule weight of the biopolymer samples. HA, ZnPc-HA, ZnPc-S-S-HA, PEG₄₀₀₀-CDM-ZnPc-HA, PEG₄₀₀₀-CDM-ZnPc-S-S-HA, PEG₄₀₀₀-CDM-ZnPc-S-S-HA were incubated under pH 6.5 at a concentration of 200 $\mu\text{g/mL}$ and then passed through a filter membrane of 200 μm , and eventually added into an aqueous column (Water 1515) with a flow rate of 1 mL/min and a temperature of 37 °C.

Flow cytometric analysis on the expression level of CD44 in MCF10 and 4T1 cells. 4T1 and MCF10 cells were seeded into 6-well plates at an initial cell density of 1×10^5 units per well. After incubating overnight, the MCF10 and 4T1 cells were digested with EDTA-Na-free trypsin solution, and washed with PBS twice before centrifugation. Fluorescent APC-CD44+ antibody was then added into the experimental group. The incubation would continue for 30 min and again washed twice with PBS before centrifugation. The fluorescence intensity of APC in each group was quantified by flow cytometry (CytoFLEX, Beckman Coulter).

Competitive HA binding assay. Similar to the set-up of the flow cytometry analysis above, 4T1 and MCF10 cells were seeded into 6-well plates at an initial cell density of 1×10^5 per well. After incubating overnight, the old media were replaced with fresh ones. Specifically, each cell type comprises two experimental groups, one is added with HA at the concentration of 200 $\mu\text{g/mL}$ and incubated for 4h before the addition of ZnPc-S-S-HA, while the other group were incubated directly with ZnPc-S-S-HA without the HA pretreatment. The incubation with ZnPc-S-S-HA would last 6 hours. When the HA-pre-treatment was complete, cells were washed with PBS for 3 times before adding ZnPc-S-S-HA. Sample concentrations were maintained at 200 $\mu\text{g/mL}$. When the incubation was complete, cells were washed with PBS for 3 times and fixed with 4% paraformaldehyde at 4 °C for 30 min, and then washed 3 times with PBS. The washed cells were mixed with rhodamine-labelled wheat germ agglutinin (10 $\mu\text{g/mL}$) and incubate overnight at 4 °C in the dark, afterwards they were washed 3 more times with PBS, and later 10 $\mu\text{g/mL}$ of H33258 (INVITROGEN) was added to stain the cell nuclei. The double stained cells were washed three times and then sealed with glycerol, and finally imaged on confocal microscope (Leica TCS SP8, Germany).

MTT assay on cell viability. The in vitro experiments in this study were carried out on mouse 4T1 breast cancer cells, which was incubated in high glucose medium supplemented with fetal bovine serum (FBS, Gibco), penicillin (100 U/mL) and streptomycin (100 $\mu\text{g/mL}$). The temperature of the cell incubator was set to 37 °C, with an atmosphere containing 5% carbon dioxide. When the cell confluence reached around 70%, they were evenly seeded into the 96-well plate at a density of 1000 cells per well. After incubation for 12 hours, the cells were washed twice with sterile PBS and then added with different samples including (1) blank control, (2) blank control + NIR, (3) PEG₄₀₀₀-CDM-ZnPc-HA, (4) PEG₄₀₀₀-CDM-ZnPc-HA + NIR, (5) PEG₄₀₀₀-CDM-ZnPc-S-S-HA, (6) PEG₄₀₀₀-CDM-ZnPc-S-S-HA + NIR, (7) ZnPc-HA, (8) ZnPc-HA + NIR, (9) weak acid-treated PEG₄₀₀₀-CDM-ZnPc-S-S-HA, and (10) weak acid-treated PEG₄₀₀₀-CDM-ZnPc-S-S-HA + NIR. Each group has 6 replicates, sample concentration was maintained at 200 $\mu\text{g/mL}$. The incubation would last for 6 hours, 12 hours

or 24 hours. Fresh medium containing MTT agent (0.5 mg/mL) was then added to each well and incubated for 4 more hours. DMSO (100 μ L) was added to each well to dissolve the emerging Formazan crystal, and the optical density was measured on a Spectra Maxi3x microplate reader under an excitation wavelength of 490 nm.

Flow cytometric analysis on sample uptake. 4T1 cells were seeded into 6-well plates at an initial cell density of 1×10^5 units per well. After incubating overnight, the old media were replaced with new ones containing PEG₄₀₀₀-CDM-ZnPc@FITC-S-S-HA, acid-treated PEG₄₀₀₀-CDM-ZnPc@FITC-S-S-HA, and blank control, and further incubated for 12 h or 24 hours. The sample concentrations were maintained at an equivalent level of 200 μ g/mL. The media were then removed, and the cells were washed once with PBS, digested with trypsin without EDTA-Na, and again washed twice before centrifugation. The fluorescence intensity of the internalized samples in each group was quantified by flow cytometry (CytoFLEX, Beckman Coulter).

CLSM study on the internalization of the biopolymer. 4T1 cells were seeded into a confocal dish at a density of 1×10^5 units per dish and incubated overnight, and then the media were replaced by new ones with blank control, PEG₄₀₀₀-CDM-ZnPc@FITC-S-S-HA, acid-treated PEG₄₀₀₀-CDM-ZnPc@FITC-S-S-HA and incubated for 6 hours, 12 hours or 24 hours. Sample concentrations were maintained at 200 μ g/mL. When the incubation was complete, cells were washed with PBS for 3 times and fixed with 4% paraformaldehyde at 4 °C for 30 min, and then washed 3 times with PBS. The washed cells were mixed with rhodamine-labelled wheat germ agglutinin (10 μ g/mL) and incubated overnight at 4 °C in the dark, afterwards they were washed 3 more times with PBS, and later 10 μ g/mL of H33258 (INVITROGEN) was added to stain the cell nuclei. The double stained cells were washed three times and then sealed with glycerol, and finally imaged on confocal microscope (Leica TCS SP8, Germany).

Quantification of the relative uptake ratio of the biopolymer. 4T1 cells were seeded into a 6-well plate at an initial density of 10^5 units per well and incubated with phenol red-free medium. After 24 hours, PEG₄₀₀₀-CDM-ZnPc@FITC-S-S-HA and ZnPc@FITC-S-S-HA were added individually into each well at 200 μ g/mL, and the total FITC fluorescence intensity in the wells was measured using a fluorescence microscope, which was denoted as A. The incubation would last 6 hours, 12 hours and 24 hours, and the medium was then removed and washed with PBS for 3 times. The purified cells were then digested with trypsin, washed with PBS for 3 times and collected via centrifugation. The total fluorescence intensity in the tumor cells was measured and denoted as B. The relative uptake ratio was calculated as B/A.

To investigate the contribution of HA-CD44 binding to the uptake of the biopolymer, 4T1 cells and MCF10 cells were seeded into 6-well plates at an initial density of 105 units per well and incubated with phenol red-free medium. After 24 hours, ZnPc@FITC-SS-HA was added into each well and the total fluorescence intensity was measured and recorded as C. After incubation for another 6 hours, the cells were equally divided into 2 groups, each comprising three wells for both cell types. The first one was added with HA at 200 $\mu\text{g/mL}$ and incubation for 2 hours. Afterwards the cells were washed 3 times with PBS and then added with ZnPc-S-S-HA (200 $\mu\text{g/mL}$) for 6 h of incubation, while the other group was directly incubated with ZnPc-SS-HA without HA pretreatment. After the incubation, the medium was removed and the cells were washed with PBS for 3 times. The purified cells were then digested with trypsin, washed with PBS for 3 times and collected via centrifugation. The total fluorescence intensity in the tumor cells was measured and denoted as D, the relative uptake ratio was calculated as D/C.

Analysis on ROS generation. 4T1 cells were seeded onto a confocal dish at a density of 1×10^5 and incubated overnight. When the cell confluence reached 70%, the cells were treated separately by (1) blank control, (2) blank control + NIR, (3) PEG₄₀₀₀-CDM-ZnPc-S-S-HA, (4) PEG₄₀₀₀-CDM-ZnPc-S-S-HA, (5) weak acid treated PEG₄₀₀₀-CDM-ZnPc-S-S-HA, and (6) weak acid treated PEG₄₀₀₀-CDM-ZnPc-S-S-HA +NIR. The laser wavelength was 720 nm with a power density of 0.5 W/cm², and the treatment was lasted 10 min. The processed cells were washed twice with PBS and then added to serum-free medium containing 10 μM DCFH-DA (Beyotime), and then incubated at 37 °C for 20 min and eventually digested. The cells were collected by centrifugation, and finally the intracellular ROS content was measured by flow cytometry (CytoFLEX, Beckman Coulter).

CLSM study on the ROS generation. 4T1 cells were seeded onto a confocal dish at a density of 1×10^5 and incubated overnight, two experiment groups were established, each containing four samples with PEG₄₀₀₀-CDM-ZnPc-HA, ZnPc-HA, PEG₄₀₀₀-CDM-ZnPc-S-S-HA and acid-treated PEG₄₀₀₀-CDM-ZnPc-S-S-HA, both at 200 $\mu\text{g/mL}$. The incubation would continue for another 24 hours, and then the cells in the second group were treated with 720 nm laser at 0.5 W/cm² for 10 min. When the treatment was complete, DCFH-DA (10 μM , Beyotime) in serum-free medium was added and the cell samples were further incubated at 37 °C for 20 min. The stained cells were washed twice with PBS, and finally analyzed by confocal microscopy (Leica TCS SP8, Germany).

Flow cytometric analysis on cell apoptosis. 4T1 cells were incubated in two 6-well plates overnight after attaching to the wall, and then 2 experiment groups were established, each

containing two samples with PEG₄₀₀₀-CDM-ZnPc-S-S-HA and acid-treated PEG₄₀₀₀-CDM-ZnPc-S-S-HA, both at 200 µg/mL. The incubation would continue for another 24 hours, and then the cells in the second group were treated with 720 nm laser at 0.5 W/cm² for 10 min, while the other group remained untreated. The incubation would continue for 2 hours, and then digested with trypsin and collected into a centrifuge tube. The collected cells were washed with PBS for 3 times and again collected by centrifugation. The purified cells were suspended into sterile PBS (200 µL), and finally stained by Annexin V/PI cell assay kit (Invitrogen). FITC (10 µL) was first added to each centrifuge tube, and PI (5 µL) was used. Apoptosis was detected by flow cytometry CytoFLEX system (Beckman Coulter).

CLSM imaging on cell live/death. 4T1 cells were incubated in a confocal dish overnight after attaching to the wall, and then two experiment groups were established, each containing four samples with PEG₄₀₀₀-CDM-ZnPc-HA, ZnPc-HA, PEG₄₀₀₀-CDM-ZnPc-S-S-HA and acid-treated PEG₄₀₀₀-CDM-ZnPc-S-S-HA at 200 µg/mL. The incubation would continue for another 24 hours, and then the cells in the second group were treated with 720 nm laser at 0.5 W/cm² for 10 min, while the other group remained untreated. The cells were gently washed twice with PBS, and then medium containing FDA fluorescein diacetate (50 µg/mL) was added for incubation for 30 min, then washed twice with PBS. Subsequently, propidium iodide (400 µg/mL) was added for incubation for 5 min in the dark and washed twice with PBS, and observed in a Leica TCS SP8 confocal laser microscope with a magnification of 40×.

Construction of the tumor spheroid models. 90 mg of agarose was firstly added into 6 mL of DMEM medium and heated under mild conditions until all the solid substances were dissolved completely. The mixture was then sterilized at 115 °C for 30 min and transferred to a super clean bench, and eventually added to a 96-well plate at 80 µL per well. Meanwhile, 4T1 cells in the exponential growth phase were digested with trypsin and added into Matrigel-supplemented DMEM medium (2.5%, v / v) at a rate of 10000 cells/mL. The cell suspensions were finally added into the agarose-containing 96-well plates and cultured in an incubator at 37 °C (5% CO₂) for 7 days to form three-dimensional 4T1 tumor spheroids. The cell culture medium is changed every 2 days. Finally, ZnPc@FITC-SS-HA, PEG₄₀₀₀-CDM-ZnPc@FITC-SS-HA (pH 7.4), PEG₄₀₀₀-CDM-ZnPc@FITC-SS-HA (culture medium supplemented with lactic acid, pH 6.5), HA+ZnPc@FITC-SS-HA were added and the treatment would continue for 12 hours. The tumor spheroids were washed with PBS for three times, placed in a confocal dish and observed with a fluorescent confocal microscope.

Western blot assay. 4T1 cells were ligated in 6-well plates overnight after attaching to the walls, and then two experiment groups were established, each containing two samples with PEG₄₀₀₀-CDM-ZnPc-S-S-HA and acid-treated PEG₄₀₀₀-CDM-ZnPc-S-S-HA, both at 200 µg/mL. The incubation would continue for another 24 hours, and then the cells in the second group were treated with 720 nm laser at 0.5 W/cm² for 10 min, while the other group remained untreated. The cells were then lysed and the total protein was extracted by centrifugation (12000 rpm for 10 min), and total protein concentration was determined by BCA protein assay kit. Protein immunoassay was performed by SDS-PAGE electrophoresis and finally photographed by a molecular imaging apparatus (Versa doc MP 4000 system, Bio-Rad).

Evaluation of the therapeutic response in vitro under acidic condition. For the evaluation of the cellular uptake, ROS generation and cell killing efficiency of PEG₄₀₀₀-CDM-ZnPc-S-S-HA under acidic conditions, the procedures were exactly the same with the cell experiments above, except that the pH of the culture medium was adjusted to 6.5 using lactic acid.

Investigation on blood circulation stability. The blood circulation stability of the biopolymers was measured using tumor-bearing balb/c mice (about 17g) as the animal model. 4T1 tumor cells were subcutaneously injected in the buttocks of the mice to establish the tumors. When the tumor development was complete, Cy5, ZnPc@Cy5-S-S-HA and PEG₄₀₀₀-CDM-ZnPc@Cy5-S-S-HA was injected into the tail vein and blood was extracted at specific time points at 0.5, 1, 2, 4, 6, 12, 24, 36 and 48h post-injection. The extracted blood samples were centrifuged at 800 rpm to remove the blood cells and the Cy5 fluorescence was determined using a fluorescence spectrometer ($\lambda_{\text{ex}} = 650\text{nm}$, $\lambda_{\text{em}} = 670\text{nm}$).

Investigations on the blood indexes. 4T1 tumor cells were subcutaneously injected into the buttocks of multiple balb/c mice (about 17g) to establish the tumor model. After that, 6 tumor-bearing mice and normal mice were selected and divided into 2 groups, each group contains three normal mice and three tumor bearing mice. The mice were then injected with PEG₄₀₀₀-CDM-ZnPc-SS-HA via the tail vein. After 12 hours, 500µl of blood was collected from the eyeball and stored in EP tube (added with anticoagulants in advance). The blood samples were well shaken and tested on the hematology analyzers (3 replicates per sample). The white blood cell count, red blood cell count, hemoglobin concentration, platelets, average red blood cell volume and average red blood cell hemoglobin volume were measured according to the user manual.

Establishing the tumor-bearing mouse model and therapeutic evaluation. Balb/c mice (6–8 weeks) were purchased from Chongqing Medical University and raised in the

Experimental Animal Center. The animal experiments in this study have been reviewed and approved by the Ethics Committees in Chongqing Medical University and carried out strictly under respective regulations. Digested 4T1 cells (100 μ L, 5×10^7 units) were subcutaneously injected into the thigh of the mice. After one week, the tumor would grow to a volume of around 50 mm³ and the mice with an average body weight of approximately 20 g. The mice were then randomly divided into 6 groups, each containing 6 replicates. The six groups were (1) blank control, (2) blank control + NIR, (3) PEG₄₀₀₀-ZnPc-S-S-HA, (4) PEG₄₀₀₀-ZnPc-S-S-HA-HA + NIR, (5) PEG₄₀₀₀-CDM-ZnPc-S-S-HA, and (6) PEG₄₀₀₀-CDM-ZnPc-S-S-HA + NIR. For the *in vivo* experiments, the samples were injected into the mice for a total of 7 times through the tail vein. After the successful establishment of the mouse tumor model, the various samples were injected on the first day and the laser illumination was 720 nm applied 24 h later. The treatment was repeated every three days. The concentration of all samples was 3 mg/kg/d. Volume of the tumors was measured via caliper, which was calculated using the following formula $V_{\text{tumor}} = L \times W^2/2$ (L: the longitudinal diameter of the tumor, W: the cross-sectional diameter of the tumor). Tumor weight was weighted using a digital balance and images were captured with a high-resolution digital camera. After 21 days of treatment, the mice were euthanized and sacrificed. Tumor and main organs including heart, liver, spleen, lung and kidney were extracted and washed with PBS. Afterwards, the main organs were fixed in 10% formalin at 4 °C for 24 h and washed with water. Moisture was removed from the tissue samples by rinsing with different concentrations of alcohol and then rinsed in toluene. The treated samples were finally embedded in wax and sliced. Some of the paraffin-embedded sections were stained with H&E (Beyotime), while others were deparaffinized with xylene and further stained with Colorimetric TUNEL Apoptosis Assay Kit (Beyotime). Both samples were imaged under a confocal microscope (Leica TCS SP8, Germany) to observe the changes of major organs and apoptosis of tumor tissues in different treatment groups.

In vivo imaging. Using the tumor-bearing mouse model established above, the mice were treated with Cy5-labelled PEG₄₀₀₀-CDM-ZnPc-S-S-HA and Cy5-labelled PEG₄₀₀₀-ZnPc-S-S-HA via intravenous injection and imaged after 6 hours, 12 hours and 24 hours on a Living-image system.

Fluorescence imaging of the tumor slices for the determination of the stimuli-responsiveness of the biopolymer in vivo. 4T1 tumor cells were subcutaneously injected into the buttocks of multiple balb/c mice (about 17g) to establish the tumor model. The FITC/Cy5 dual-labeled FITC-PEG₄₀₀₀-CDM-ZnPc@Cy5-S-S-HA biopolymer was injected through the tail vein and the mice were sacrificed 12 h after administration. The tumor tissues

were extracted and sliced. The cell nuclei were stained with H33258 for 10 min and washed with PBS for 3 times before being mounted on glass slides. The distribution patterns of the green and red fluorescence were observed using CLSM.

Kaplan-Meier survival curve. Mice with established tumor were randomly divided into 6 groups, each comprising 6 replicates. The 6 groups were treated separately with (1) blank control, (2) blank control + NIR, (3) PEG₄₀₀₀-ZnPc-S-S-HA, (4) PEG₄₀₀₀-ZnPc-S-S-HA + NIR, (5) PEG₄₀₀₀-CDM-ZnPc-S-S-HA, and (6) PEG₄₀₀₀-CDM-ZnPc-S-S-HA + NIR, which were injected via the tail vein. The sample concentration was maintained at 3mg/kg/d. The 720 nm laser illumination was exercised 24 h after the first sample injection, the mouse survival was recorded on a daily basis.

Supporting Information

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

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Scheme 1: Chemical structure of the ZnPc-incorporated biopolymer and schematic representation of self-assembly-enabled tumor-targeting and sequentially responsive ZnPc delivery mechanisms. The ZnPc moiety was marked with a dotted frame as the conjugation reactions may occur randomly with one of the amino groups.

Figure 1. TEM images of (a) PEG₄₀₀₀-CDM-ZnPc-S-S-HA under neutral conditions and (b) PEG₄₀₀₀-CDM-ZnPc-S-S-HA in PBS at pH 6.5. The inserted image in panel (b) shows the Tyndall effect of the sample. (c) Scanning transmission electron microscopy image of PEG₄₀₀₀-CDM-ZnPc-S-S-HA in PBS at pH 6.5. (d) Absorption and fluorescence spectra of PEG₄₀₀₀-CDM-ZnPc-S-S-HA (black), ZnPc-S-S-HA (red) and ZnPc (blue) at the same concentration. (e) Size distribution of PEG₄₀₀₀-CDM-ZnPc-S-S-HA under neutral pH and pH 6.5, characterized by dynamic light scattering. (f) ZnPc release profiles from PEG₄₀₀₀-CDM-ZnPc-S-S-HA under pH 6.5+10 mM GSH, pH 7.4+10 mM GSH, pH 7.4 and pH 6.5.

Figure 2. (a,b) Fluorescence imaging on the endocytosis of (a,a1,a2) PEG₄₀₀₀-CDM-ZnPc@FITC-S-S-HA and (b,b1,b2) acid-treated PEG₄₀₀₀-CDM-ZnPc@FITC-S-S-HA by tumor cells after different incubation time under FITC/H33258 dual fluorescence channels. The incubation periods were set as 6h, 12h and 24h, which corresponded to sample group a/b, a1/b1 and a2/c2, respectively. (c) Quantitative FITC fluorescence intensity of the three groups in (a-c). (d) Flow cytometric results regarding the cellular uptake of the three groups under 24h. (e-e1) Uptake of ZnPc@FITC-S-S-HA by MCF-10 cells with or without HA pretreatment. (f-f1) Uptake of ZnPc@FITC-S-S-HA by 4T1 cells with or without HA pretreatment. (g) Total FITC fluorescence in panel e/e1/f/f1. (h) Expression level of CD44 in MCF10 and 4T1 cell lines, measured via flow cytometry with APC-CD44⁺ antibody assay kit.

Figure 3. Penetration of various biopolymer samples in 4T1 tumor spheroids. The fluorescence images were captured consecutively at a depth interval of 40 μm . Scale bar: 150 μm . The treatments include: (I) ZnPc-S-S-HA, (II) PEG₄₀₀₀-CDM-ZnPc-S-S-HA at pH 7.4, (III) PEG₄₀₀₀-CDM-ZnPc-S-S-HA at pH 6.5 (culture medium was supplemented with lactic acid), and (IV) ZnPc-S-S-HA+HA.

Figure 4. (a) ROS generation in the sample-treated tumor cells with or without NIR irradiation, studied using DCF-DA assay kit that emits strong green fluorescence after reacting with environmental ROS. (b) Quantitative FITC fluorescence intensity of the samples in panel a. (c) DCF-DA-enabled flow cytometric analysis on the ROS generation in 4T1 cells in each group.

Figure 5. (a) Representative CLSM live/dead cell images of 4T1 cells dual-stained by Calcein-AM and propidium iodide (PI) with or without NIR treatment. (b) Flow cytometric

analysis of cell apoptosis after different treatments with (I) PBS, (II) PEG₄₀₀₀-CDM-ZnPc-S-S-HA, (III) ZnPc-S-S-HA, (IV) PBS + NIR, (V) PEG₄₀₀₀-CDM-ZnPc-S-S-HA + NIR, and (VI) ZnPc-S-S-HA + NIR. (c) Western blotting analysis on the expression levels of Bcl-2, Bax and α -Tubulin in 4T1 cells after treatments with (I) PBS, (II) PEG₄₀₀₀-CDM-ZnPc-S-S-HA, (III) ZnPc-S-S-HA, (IV) PBS + NIR, (V) PEG₄₀₀₀-CDM-ZnPc-S-S-HA + NIR, and (VI) ZnPc-S-S-HA + NIR, respectively. (d) Dot plots of the flow cytometric results on cell apoptosis after different treatments with (I) PBS, (II) PEG₄₀₀₀-CDM-ZnPc-S-S-HA, (III) ZnPc-S-S-HA, (IV) PBS + NIR, (V) PEG₄₀₀₀-CDM-ZnPc-S-S-HA + NIR, and (VI) ZnPc-S-S-HA + NIR.

Figure 6. Fluorescence imaging on the distribution pattern of different biopolymer samples in 4T1-tumor-bearing rodent models. (a,b) Whole-body distribution of PEG₄₀₀₀-ZnPc-S-S-HA and PEG₄₀₀₀-CDM-ZnPc-S-S-HA at 6/12/24 h post intravenous injection, respectively. (c,d) Corresponding quantitative fluorescence in major organs and tumors. The small white edges in the images are due to the white body hairs of the Balb/c mice.

Figure 7. (a) Visual comparison of tumors extracted from the mice in each group. The scale bar represents 1 cm. (b) Changes in the tumor volumes for each group throughout the treatment period. (c) Kaplan-Meier curve on the mouse survival in each group. (d) H&E staining and TUNEL staining of tumor tissue sections after the treatment with (I) PBS, (II) PBS+NIR, (III) PEG₄₀₀₀-ZnPc-S-S-HA, (IV) PEG₄₀₀₀-ZnPc-S-S-HA + NIR, (V) PEG₄₀₀₀-CDM-ZnPc-S-S-HA, and (VI) PEG₄₀₀₀-CDM-ZnPc-S-S-HA + NIR.

A sequentially responsive biopolymer integrated with zinc phthalocyanine (ZnPc) is developed for tumor-targeted photodynamic therapy, which could self-assemble in acidic tumor tissues in a self-regulated manner to achieve enhanced tumor retention and uptake, leading to the ZnPc release in the tumor intracellular environment for increased photodynamic efficacy.

Keyword: photodynamic biopolymer, self-assembly, sequential responsiveness, targeted drug delivery, tumor microenvironment

Xuan Wang, Menghuan Li, Yanhua Hou, Yanan Li, Xuemei Yao, Chencheng Xue, Yang Fei, Kaiyong Cai, Yanli Zhao, Zhong Luo**

Tumor-microenvironment-activated *in situ* self-assembly of sequentially responsive biopolymer for targeted photodynamic therapy