Size Switchable Nanoclusters Fueled by Extracellular ATP for Promoting Deep Penetration and MRI-Guided Tumor Photothermal Therapy

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Protein-based theranostic agents (PBTAs) exhibit superior performance in the diagnosis and therapy of cancers. However, the in vivo applications of PBTAs are largely limited by undesired accumulation, penetration, or selectivity. Here, an ATP-supersensitive protein cluster is fabricated for promoting PBTAs delivery and enhancing magnetic resonance imaging (MRI)-guided photothermal therapy. Gd³⁺ and CuS-coloaded small bovine serum albumin nanoparticles (GdCuB) are synthesized as the model protein with a size of 9 nm and are encapsulated into charge switchable polycations (DEP) to form DEP/GdCuB nanoclusters of 120 nm. In blood circulation, DEP/GdCuB significantly extends the half-lifetime and thereby enhances the tumor accumulation of GdCuB. When the clusters reach the tumor site, the extracellular adenosine triphosphate (ATP) can effectively trigger the release of GdCuB, resulting in tumoral deep penetration as well as the activation of T₁-weighted MRI (T₁ value switched from 2.8 x 10⁻³ to 11.8 x 10⁻³ m⁻¹ s⁻¹). Furthermore, this delivery strategy also improves the tumoral photothermal therapy efficacy with the MRI-guided therapy. The study of ATP-activated nanoclusters develops a novel strategy for tumor deep penetration and on/off imaging of PBTAs by size switchable technology, and reveals the potential for MRI-guided therapy of cancers.

1. Introduction

In the past few decades, proteins or protein-based theranostic agents (PBTAs) have been extensively explored for the diagnosis and treatment of various cancers.[1] Therapeutic proteins, such as GOx,[2] DNase I,[3] RNase A,[4] cytochrome c,[5] or β-Gal,[6] as bio-macromolecular drugs, exhibited exciting activity on antitumor therapy. Other biocompatible proteins, like albumin,[6] hemoglobin,[7] or transferrin,[8] could acted as the carrier of therapeutic small molecular drugs, dyes or the diagnosis agent. However, the application of these PBTAs was largely bottlenecked by the dissatisfied accumulation, penetration, or selectivity in tumor for the uncontrollable size, shape, surface properties, or activities. Therefore, the ameliorating of the in vivo delivery performance of PBTAs was still a challenge for researchers.

Among all of these properties, particle size is one of the key factors that affect the blood circulation time, tumor accumulation and tumor penetration of nanoparticles, as well as the enzyme activity or imaging effect.[9] The diameter of the recently reported PBTAs was mainly around 4–300 nm.[10] The particles with smaller size exhibited higher surface to volume ratio, resulting in the more water molecules coordinated to the metal ions (Gd, Fe, or Mn), obtaining enhanced T₁-weighted magnetic resonance imaging (MRI) effect.[9] In addition, it was demonstrated that the nanoparticles with smaller particle size could achieve deeper tumor penetration because of the reduced diffusional obstacle.[10] However, the smaller size often means limited circulation half-lifetime and inefficient tumor accumulation. The particles with diameter less than 10 nm were often observed to be rapidly eliminated by renal. Besides, the size smaller than 50 nm was more easily to interact with hepatocytes and be filtered by the liver.[11] On the contrast, particles with size around 100 nm not only have the optimal performance in vivo administration with prolonged circulation time in the body, but also the effective tumor accumulation by EPR effect.[12] Unfortunately, the nanoparticles with size around 100 nm displayed poor penetration efficiency in solid tumors compared particles with the smaller size.[13] Therefore, it raised an important concern that maintaining the balance of accumulation and penetration was still a considerable obstacle for achieving desired treatment efficacy or imaging resolution.

Over the years, smart nanodelivery systems have received tremendous attentions for stimulus-triggered assembly/disassembly with timely and spatially controlled manner.[14] It was worth noting that the dynamic switch of the particle structures could initiate the “on–off” effect of diagnosis and therapy, enhancing the resolution of diagnosis and reducing the side effects of therapy.[15] However, the recent studies were mostly focused on the acidic...
environment of tumor (pH 5.5–7.2). Few people paid attention to the relative abundant adenosine triphosphate (ATP) in the extracellular environment of tumor (1 × 10^{-4} to 4 × 10^{-4} m), especially for deep penetration study. Actually, the concentration of tumor extracellular ATP is much higher than normal tissues (1 × 10^{-9} to 10 × 10^{-9} m), enabling it be exploited as the selective trigger for stimulus-responsive delivery system construction. Furthermore, our previous study demonstrated that the pretty high concentration of intracellular ATP (1 × 10^{-3} to 10 × 10^{-3} m) could significantly drive the charge reversal of phenylboronic acid (PBA) functional polycations and result in the release of negatively charged siRNA. Here, in this study, we would like to further expand the role of extracellular ATP as a trigger in modulating assembly/disassembly of PBTA nanoclusters and thereby facilitating tumor penetration and on/off imaging of PBTA.

This study is aimed at exploiting ATP-activated permeable nanocluster for MRI-guided tumor photothermal therapy (PTT). We first prepared the Gd^{3+} and CuS-coloaded small bovine serum albumin (BSA) nanoparticles (GdCuB) as the model protein equipped with both MRI and PTT functions. Subsequently, PBA and ethylenediamine (EDA) decorated dextrin (DEP) was synthesized and worked as the charge switchable polycationic carrier. The small GdCuB was encapsulated into DEP to form DEP/GdCuB cluster. As shown in Scheme 1, we made the hypothesis that a) in blood circulation, the relative larger size of DEP/GdCuB would extend the half-lifetime of GdCuB and hence enhance the tumor accumulation. Besides, the formation of cluster weakened the \( T_1 \)-weighted MRI effect of GdCuB, reducing the background signal; b) at tumor site, the extracellular ATP triggered the release of GdCuB, promoting the deep penetration of clusters as well as activating the \( T_1 \)-weighted MRI of GdCuB. The deep penetration of photothermal agent GdCuB plus with 1064 nm laser would also strengthen the anticancer efficacy. The hypothesis of the size switchable protein cluster activated by extracellular ATP would significant enhance the MRI diagnosis guided PTT therapy of tumor simultaneously. It also provided a strategy for ameliorating the in vivo performance of PBTA.

### 2. Result and Discussion


The synthesis procedure of dextrin–EDA–PBA (DEP) was shown in Figure S1 (Supporting Information). Ethylenediamine was introduced to dextrin by the catalysis of CDI to obtain a biodegradable polycations (dextrin–EDA). The chemical structures were validated by \(^1\)H-NMR. As shown in Figure S2 (Supporting Information), the peaks among 2–3 ppm was ascribed to the methylene of EDA, which prove the successful amidation of dextrin. The modified ratio of EDA to sugar ring was 52%. Phenyboronic acid was next modified on Dex–EDA by amidation reaction. The peaks among 7–8 ppm was ascribed to the hydrogen of benzene, which was used to compare with the peak of hydroxyl. The PBA modification ratio was 13.6% and 29.7% for DEP1 and DEP2 compared with sugar ring of dextrin. The successful synthesis of Dex–EDA and DEP was also confirmed by FTIR (Figure S3, Supporting Information), demonstrated as a characteristic peak of 1704.2 cm^{-1} of carbonyl group on Dex–EDA and a characteristic peak of 1648.9 cm^{-1} of phenyl group on DEP.

#### 2.2. Preparation and Characterization of GdCuB Nanoparticles and DEP/GdCuB Nanocluster

The GdCuB was synthesized by biomineralization strategy and used as a model protein drug, which was equipped with both...
photothermal effect and MRI effect. The particle size of GdCuB detected by DLS was 11.8 nm and zeta potential was $-12.8$ mV, which met up the requirement of this study.

To verify the successful encapsulation of GdCuB by DEP, GdCuB was labeled with FITC. As shown in Figure 1A, with the increasing DEP introduced, the fluorescence intensity of FITC at 520 nm emission decreased, indicating the successful encapsulation of GdCuB-FITC in DEP. Next, the particle size of DEP/GdCuB was measured by DLS as 120 nm (Figure 1B), proving that GdCuB was loaded in DEP to form a cluster. With the cationic DEP encapsulation, the zeta potential of DEP/GdCuB reversed to positive as $13.7$ mV compared with the negatively charged GdCuB (Figure 1C).

The serum stability of the nanoclusters was evaluated by monitoring the size after incubation with serum. As shown in Figure S4 (Supporting Information), the size could be relatively stable at about 120 nm within 48 h, where the size changing range was less than 15 nm. It was shown as a moderate increasing among 0–6 h from 116.3 to 128.5 nm and keeping steady among 6–48 h. The result demonstrated the good serum stability of the DEP/GdCuB and the potential for in vivo application.

2.3. ATP-Triggered Charge Reversal of DEP and Release of GdCuB Nanoparticle

In our previous study, we find that intracellular ATP could effectively triggered the charge reversal of PBA functional polycations, leading to the release of negatively charged siRNA. Similarly, BSA is also negatively charged macromolecule (PI 4.6). Therefore, we are desired to research whether ATP could effectively trigger the release of BSA nanoparticles from the cluster. As we know, the extracellular concentration of ATP of tumor is $1 \times 10^{-4}$ to $4 \times 10^{-4}$ m, which is pretty lower than intracellular environment ($1 \times 10^{-3}$ to $10 \times 10^{-3}$ m). It was of great important to improve the sensitivity of the carriers. Here, an increasing PBA modification ratio of Dex–EDA–PBA (DEP0 means 0%, DEP1 means 13.6%, DEP2 means 29.7%) was synthesized to optimize the best ratio for further research. The hypothesis of
ATP-triggered charge reversal was shown in Figure 1D. The DEP was positively charged while ATP was negatively charged. When the DEP was exposed in the ATP abundant environment, the dynamic chemical reaction between the PBA and ATP could significantly promote the ATP binding on DEP, which resulted in the charge reversal of DEP from positive to negative by the introduction of phosphates. Taking this hypothesis in mind, we evaluated the zeta potential changing of DEP with the increasing ATP involvement (0–4 × 10^{-4} m). As shown in Figure 1E, without PBA modification, DEP0 exhibited weak ATP sensitivity, exhibited as mild changing of zeta potential with the increasing ATP added. Expectantly, DEP2, with high PBA modification ratio, was pretty sensitive to ATP. The zeta potential of DEP2 decreased sharply and was even reversed to negative charge when the ATP concentration was as high as 1 × 10^{-4} m, which validated our hypothesis that the PBA modification could improve the sensitivity of DEP to ATP.

The ATP-triggered charge reversal of DEP reminded us to suppose that the charge reversal may result in the GdCuB release since it was loaded into the cluster by electrostatic interaction. Therefore, the release behavior of GdCuB from various clusters (DEP0/GdCuB, DEP1/GdCuB, and DEP2/GdCuB) was further studied by the fluorescence recovery method with the ATP involvement. As shown in Figure 1F, dextrin–EDA (DEP0) based cluster was insensitive to the ATP from 1 × 10^{-4} to 4 × 10^{-4} m, nearly no GdCuB-FITC released. On the contrast, PBA functional dextrin–EDA exhibited dramatically responsive to ATP, where the responsive ability increased with the more PBA conjugated. About 76% GdCuB-FITC released from DEP2/GdCuB-FITC with the treatment of relative low concentration ATP of 1 × 10^{-4} m within 30 min, displayed the supersensitivity of DEP2/GdCuB to ATP. Hence, DEP2 was chosen for further research. DEP represented DEP2 through the full text.

The ATP-triggered disassembly of cluster and release of GdCuB was also demonstrated by FRET technology. FITC was labeled on GdCuB (GdCuB-FITC) which acted as the donor and rhodamine B (RhB) was labeled on DEP (DEP–RhB) as the acceptor. As shown in Figure 1G, without ATP treatment, the energy of FITC could effectively transfer to RhB, shown as the appearance of both emissions of FITC and RhB with 488 nm excitation. However, when increasing ATP was involved in the system, the emission peak of RhB decreased sharply, indicating the release of GdCuB from DEP/GdCuB clusters.

The morphology observation of GdCuB, DEP/GdCuB with or without 1 × 10^{-4} m ATP treatment was also performed. As shown in Figure 1H, the average size of GdCuB and DEP/ GdCuB was around 90 and 100 nm, respectively, which was consistent with the DLS detecting result. Exhilaratingly, with the ATP treatment, many small particles release from the big clusters, further validating the result in FRET experiment. The size and morphology of released small nanoparticles were similar with the GdCuB as around 9 nm. Here, the ultrasmall size and negative charge of GdCuB were extremely beneficial for tumor deep penetration as well as the T1-weighted MRI owing to the high surface to volume ratio.

It could be concluded that 1 × 10^{-4} m ATP could effectively trigger the charge reversal of DEP and lead to the release of GdCuB from clusters. Besides, the increasing of PBA modification ratio was demonstrated to improve the responsive capability of DEP to obtain ATP supersensitive clusters, enabling the on-demand release of GdCuB in the extracellular environment.

### 2.4. Photothermal Effect and Tumor Cell Killing

The UV–vis spectrum of GdCuB and DEP/GdCuB was also detected. Both GdCuB and DEP/GdCuB had strong absorbance at 1064 nm (Figure 2A). Therefore, we chose 1064 nm laser (NIR II) for photothermal study, which exhibited deeper tissue penetration capability compared with traditional NIR I laser. The GdCuB or DEP/GdCuB solution was exposure under 1064 nm laser and the temperature of the solution was monitored. As shown in Figure 2B and Figure S5 (Supporting Information), after laser irradiation, the temperature of GdCuB rise sharply and reached 56 °C at 120 s measured by both IR cameral or temperature detector. The final temperature of DEP/GdCuB cluster was a little lower than GdCuB because of the slightly weaker absorption at 1064 nm.

Subsequently, the tumor cell killing effect of GdCuB and DEP/GdCuB after laser irradiation was evaluated on 4T1 cells by MTT assay. As shown in Figure 2C, without laser irradiation, both GdCuB and DEP/GdCuB did not cause obvious cell toxicity, meaning good biocompatibility of the nanomaterials. Expectantly, after laser treatment, they cause violent tumor cell killing effect, indicating the strong potential for tumor treatment.

Live/dead cell staining was also performed to validate the tumor cell killing effect of GdCuB and DEP/GdCuB after laser irradiation was evaluated on 4T1 cells by MTT assay. As shown in Figure 2C, without laser irradiation, both GdCuB and DEP/GdCuB did not cause obvious cell toxicity, meaning good biocompatibility of the nanomaterials. Expectantly, after laser treatment, they cause violent tumor cell killing effect, indicating the strong potential for tumor treatment.

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**2.5. ATP-Activated Fluorescence Imaging and Magnetic Resonance Imaging**

Most of the traditional fluorescence probes have the behavior of aggregation caused quenching (ACQ). It was also observed that the encapsulation with DEP could lead to the fluorescence quenching of FITC labeled GdCuB nanoparticles (Figure 1A). Next, we studied the fluorescence recovery of GdCuB-FITC after ATP treatment. As depicted in Figure 2D, without ATP treatment, the fluorescence intensity of DEP/GdCuB-FITC was low. However, with the increasing ATP involvement from 0 to 1 × 10^{-4} m, the fluorescence intensity of FITC was enhanced from 200 to 700, proving the ATP-triggered recovery of fluorescence. In addition, we also labeled GdCuB with NIR probe Cy7. DEP/GdCuB-Cy7 nanoclusters were treated with various concentration of ATP and the fluorescence images of all the samples were obtained by Caliper IVIS Lumina II in vivo image system. The result exhibited that the fluorescence signal was much higher after ATP treatment compared with untreated sample (Figure 2E). It means the DEP/GdCuB-Cy7 system held great potential to be used for tumor specific amplified fluorescence imaging.
The assembly and disassembly of MRI particles often resulted in the altering of the longitudinal ($T_1$) and transverse ($T_2$) relaxation times. As displayed in Figure 2F, the GdCuB solution exhibited obvious brightening effect imaged in $T_1$ model with the increasing concentration of Gd$^{3+}$. However, the brightening effect weakened after DEP encapsulation. Interestingly, with the ATP treatment, it recovered again and the intensity was as high as the free GdCuB nanoparticles, indicating the ATP could light the $T_1$-weighted MRI of DEP/GdCuB. The darkening effect imaged in $T_2$ model was shown in Figure S7 (Supporting Information). The longitudinal ($r_1$) and transverse ($r_2$) relaxation rates of GdCuB were detected to be $11.785 \times 10^{-3}$ m$^{-1}$ s$^{-1}$ (Figure 2G) and $32.258 \times 10^{-3}$ m$^{-1}$ s$^{-1}$ (Figure S7, Supporting Information) respectively. Here, the $r_2/r_1$ value was 2.74 (<3), indicating that GdCuB was a promising $T_1$-weighted contrast agent.

As we know, the number of water molecules directly coordinated to the paramagnetic metal ions was one of the key roles that affected the longitudinal relaxation. The high $r_1$ value of GdCuB could be ascribed to the ultrasmall particle size of around 9 nm, which provided a high surface to volume ratio for sufficient water–metal interactions, resulting in the shortened longitudinal relaxation times. Expectably, after encapsulated with DEP, the $r_1$ value dropped sharply to $2.8041 \times 10^{-3}$ m$^{-1}$ s$^{-1}$, which could be owing to the reduced surface to volume ratio of DEP/GdCuB compared with free GdCuB. Interestingly, the ATP treatment could effectively induce the recovery of $r_1$ value to $9.6998 \times 10^{-3}$ m$^{-1}$ s$^{-1}$, ascribing to the triggered release of GdCuB from cluster. Moreover, the $r_2$ value shares the same tendency as $r_1$. After encapsulated with DEP, the $r_2$ value decreased to $13.02 \times 10^{-3}$ m$^{-1}$ s$^{-1}$, followed by rising to $28.432 \times 10^{-3}$ m$^{-1}$ s$^{-1}$ after ATP treatment. The presented result demonstrated that the DEP/GdCuB had satisfactory performance on ATP activated MR imaging, especially used as $T_1$-weighted contrast agent.

2.6. ATP-Activated Tumor Deep Penetration

Many papers proved that nanoparticles with smaller sizes exhibited enhanced tumor penetration. However, particles with small sizes, especially less than 10 nm, are also easily to
be cleared by renal, leading to reduced tumor accumulation. Therefore, maintaining relative larger size in the blood circulation, but switching to small size at tumor site was a reliable strategy for enhancing the tumor accumulation and penetration simultaneously.

The supersensitivity of the nanoclusters to ATP treatment had been already validated by the FRET and TEM analysis. Next, we were desired to check whether the release of GdCuB nanoparticles could promote the tumor penetration. The in vitro ATP-triggered penetration was evaluated on 4T1 multicellular spheroids (MCSs). DEP/GdCuB-FITC were incubated with MCSs in the presence or absence of ATP for 12 h and monitored by CLSM. As shown in Figure 3A, without ATP treatment, the DEP/GdCuB-FITC cluster showed weak penetration on 4T1 MCSs, where the fluorescence signal was mainly distributed on the periphery of the spheroids. Expectantly, after incubation with $1 \times 10^{-4}$ M ATP, the penetration depth was evidently increased to around 60 µm, demonstrating that the release of GdCuB-FITC small nanoparticles could effectively promote the tumor penetration. The 2.5D analysis of the confocal images at 60 µm depth displayed the strong distribution of fluorescence signal in the spheroids (Figure 3B). The ATP-triggered rapid release of GdCuB could significantly enhance the distribution of the theranostic nanoparticles in the tumor tissue. Besides, the deep penetration of GdCuB-FITC increased the opportunities of cell uptake by cancer cells. As analyzed by flow cytometry (Figure 3C), for ATP treatment group, the ratio of FITC positive cells was pretty higher than untreated group, around fourfolds of untreated group. It should also be noted that the penetration was fast, where the positive cell ratio at 4 h was almost the same level as 12 h, which could be ascribed to the rapid responsive of nanocluster to extracellular ATP and the extremely small particle size of GdCuB released. Meanwhile, the mean fluorescence intensity of ATP treated DEP/GdCuB was much stronger than ATP untreated group (Figure 3D). To conclude, the DEP/GdCuB cluster played good performance on 4T1 MCSs model in vitro, rapid penetrating into deep tumor spheroids.

The in vitro penetration experiment was performed on MCSs model by mimicking the tumor ATP environment of $1 \times 10^{-4}$ to $4 \times 10^{-4}$ M ATP. The penetration behavior was further studied in vivo to check whether it work in the body. Here, 4T1 tumor model was constructed on BALB/c mice for in vivo evaluation. The red fluorescence of blood vessels stained by CD31 antibody and green fluorescence of GdCuB-FITC were observed by CLSM at the depth of 2 mm. As shown in Figure 3E, both free

![Figure 3](image-url)

**Figure 3.** A) Tumor penetration of DEP/GdCuB-FITC with or without $1 \times 10^{-4}$ M ATP treatment on 4T1 MCSs observed by CLSM z-stack ($n = 3$); scale bar: 100 µm. B) 2.5D analysis of the confocal images at 60 µm depth ($n = 3$). C) FITC positive cells and D) MFI analysis by FCM after 4 h or 12 h treatment with DEP/GdCuB-FITC with or without $1 \times 10^{-4}$ M ATP (mean ± SD, $n = 3$). E) Confocal images of frozen tumor sections ($n = 3$). Tumor blood vessels were labeled with Cy3–CD31 antibody (red). FITC is shown in green and the nucleus in blue (Hoechst 33258); scale bar: 100 µm.
GdCuB-FITC and DEP/GdCuB-FITC could penetrate into the deep tumor, displaying as the dispersive green signal in the tumor frozen sections. Moreover, the green fluorescence signal of DEP/GdCuB-FITC was pretty stronger compared with GdCuB-FITC group, indicating the enhanced accumulation of DEP/GdCuB-FITC. The DEP/GdCuB-FITC clusters may act as a smart depot of GdCuB-FITC, which enabled the accumulation and penetration of nanoparticles simultaneously. All of the results confirmed that the DEP/GdCuB-FITC could effectively penetrate into the deep tumor with the stimulus of the extracellular ATP.

2.7. In Vivo NIRF Imaging, Biodistribution and Blood Circulation Behavior

The cluster was also endowed with near-infrared fluorescence (NIRF) imaging ability by labeling GdCuB with NIRF probe Cy7. The Cy7 could track the biodistribution of nanoparticles in the tumor bearing mice by Caliper IVIS Lumina II in vivo image system. As shown in Figure 4A, at 12 h postinjection, the fluorescence signal was mainly distributed in the liver for both GdCuB-Cy7 and DEP/GdCuB-Cy7, while the signal of DEP/GdCuB-Cy7 at tumor site was stronger than GdCuB-Cy7. The maximum accumulation of DEP/GdCuB-Cy7 was observed at 24 h postinjection, which was much higher than GdCuB-Cy7. The result exhibited the size switching strategy on improving the tumor accumulation of nanoparticles. The strong accumulation of nanoclusters at tumor site was also validated by detecting the fluorescence of tissues lysate at 24 h postinjection (Figure 4B). The accumulation amount of DEP/GdCuB-Cy7 group was about 3.2 times of GdCuB-Cy7 group.

The preparing nanoclusters was also desired to extend the blood circulation time of GdCuB. As shown in Figure 4C, the free GdCuB was quickly eliminated from the body, the half-life-time ($t_{1/2}$) was around 1 h. Interestingly, the clusters showed a slower clearance ratio and the $t_{1/2}$ of DEP/GdCuB-Cy7 was around 5 h, much longer than the free GdCuB nanoparticles. The clusters were demonstrated to significantly improve the
blood circulation time of the PBTA within 24 h and hence increase the tumor accumulation. Moreover, almost all of the clusters could be eliminated from the body within 48 h, indicating the clearable property of the clusters.

Therefore, it could be concluded that the prompt accumulation of DEP/GdCuB–Cy7 at tumor site could be owing to the extensive blood circulation time, passive targeting by EPR and active targeting of PBA to tumor cells (sialic acid overexpression).

2.8. In Vivo MR Imaging and Photothermal Imaging

The in vitro result demonstrated that the GdCuB held great potential to be applied for in vivo $T_1/T_2$-weighted MRI. In addition, the on-off imaging effect was also validated in the solution samples. Therefore, we further evaluated the in vivo MRI behavior on 4T1 tumor model. Briefly, the GdCuB or DEP/GdCuB formulations were administrated by i.v. injection and the MR imaging was performed at preinjection, 6 and 24 h after injection. As depicted in Figure 4D, the $T_1$-weighted MRI signal of DEP/GdCuB treated mice was shown as significant brightening effect at 6 and 24 h postinjection compared with preinjected mice, as well as the clearer edge of the tumor. Besides, it should also be mentioned that the tumor exhibited strong darkening effect observed at $T_2$-weighted MRI model. It reflected that DEP/GdCuB was a superior contrast agent for tumor $T_1/T_2$ MRI. On the contrary, the MRI effect of GdCuB was much weaker than DEP/GdCuB, probably because of the inefficient tumor accumulation of GdCuB. These results proved that the preparing ATP-activated permeable cluster was an efficient strategy for improving the in vivo imaging effect of traditional PBTA.

In this PBTA, CuS was encapsulated in the GdCuB to initiate photothermal effect at tumor site. The high resolution of MRI could help to guide the treatment of laser irradiation at tumor site. Here, we also chose a NIR II laser of 1064 nm for enhancing the organ permeating depth. The tumor temperature rising by the laser was observed by IR camer. As shown in Figure 4E, the tumoral temperature of saline treatment group rise slightly after 4 min laser irradiation. Differently, the temperature increased dramatically with the laser irradiation after administration of GdCuB or DEP/GdCuB, where the DEP/GdCuB showed better performance. The better photothermal effect of DEP/GdCuB could be ascribed to the enhanced tumor accumulation of CuS. The tumor temperature could reach as high as 55 °C, which was sufficient to directly kill the tumor cells.[27]

The above results proved that the ATP-activated permeable strategy significantly enhance the imaging effect of GdCuB by MRI, NIRF, or photothermal imaging.

2.9. In Vivo Antitumor Efficacy

It was demonstrated that laser irradiation could effectively initiate the temperature rising at tumor site as shown above. We further studied the anticancer efficacy of photothermal treatment. The tumor bearing mice were given different treatment every 4 days. The tumor volume and body weight of mice were monitored. As depicted in the result (Figure 5A), saline + laser treatment or DEP/GdCuB treatment did not exhibited any tumor suppression activity. The photothermal effect on tumor cell killing generally worked when the temperature reached as high as 43 °C. However, the laser irradiation of saline treated mice could not induce significant temperature increasing (less than 43 °C), leading to the invalid antitumor efficacy of saline + laser treatment. Besides, the injection of nanomaterial DEP/GdCuB without laser also had no tumor suppression efficacy. The administration of GdCuB small nanoparticles plus 1064 nm laser irradiation effectively inhibited the tumor growth, about 45% inhibition versus saline group. Excitingly, the DEP/GdCuB plus laser irradiation significantly inhibited the tumor growth and even caused the tumor ablation, showing extreme antitumor efficacy. It could be ascribed to the effective tumor accumulation, and deep penetration of the photothermal agent, which finally resulting in better photothermal therapy compared with free GdCuB treatment group. The representative tumors were imaged and depicted in Figure 5B. The superiority of the delivery platform was also confirmed by weighting the tumor weight (Figure 5C), which was consistent with the tumor growth curve. The tumor weight of GdCuB + 1064 nm was 41% versus saline group and DEP/GdCuB + 1064 nm was rarely to be detected, indicating the cluster could achieving fantastic antitumor therapy due to the enhancement of GdCuB delivery. Meanwhile, no obvious weight loss was found of all the groups compared with saline treatment group (Figure 5D), indicating good biocompatibility of the model theranostic agent of GdCuB and the nanocluster delivery platform of DEP/GdCuB.

The photothermal induced tumor apoptosis and necrosis was studied by H&E staining and TUNEL staining (Figure 5E). No notable necrosis was found in these three groups (saline, saline + 1064 nm, and DEP/GdCuB). Expectantly, GdCuB + 1064 nm treated tumors appeared clear necrosis and karyolysis in the sections.[28] Furthermore, the necrosis area of DEP/GdCuB + 1064 nm was much larger than GdCuB + 1064 nm treatment group, which explained the better antitumor efficacy of the cluster. TUNEL training analysis was also applied to study the photothermal induced cell apoptosis. The nuclei was stained with DAPI and FITC labeled dUTP was used to stain the fragmented DNA. The CLSM images demonstrated that the GdCuB + 1064 nm significantly induced cell apoptosis, shown as stronger green fluorescence versus saline group. Dramatically, the DEP/GdCuB + 1064 nm treated group exhibited a much brighter fluorescence compared with GdCuB + 1064 nm group, meaning more serious apoptosis and necrosis.

Therefore, these results demonstrated the benefits of the ATP-activated permeable strategy on enhancing the antitumor efficacy of small nanoparticles.

2.10. Biosafety Study

The biosafety of the theranostic agents are very important for successful in vivo application. After 4 treatments, the main organs of the mice were harvested and performed by H&E staining. As shown in Figure S8 (Supporting Information), no obvious cytotoxicity was found in the main organs of both GdCuB group and DEP/GdCuB group compared with saline treated mice, indicating the excellent biocompatibility of the GdCuB and DEP/GdCuB.
3. Conclusion

In this work, an ATP-activated permeable nanocluster was successfully fabricated for MRI diagnosis guided photothermal therapy of breast cancer. Gd³⁺ and CuS coloaded BSA nanoparticle with a size of 9 nm was used as the model PBTA. The ATP-supersensitive polycations of DEP effectively modulated the assembly and disassembly of nanoclusters with or without the ATP treatment. After i.v. injection of DEP/GdCuB, the cluster obviously reduced the clearance rate of GdCuB, enhancing the tumor accumulation of GdCuB by EPR Effect. The formation of cluster also shielded the MRI effect of GdCuB, weakening the background signal in the circulation. Upon reaching the tumor site, the extracellular ATP promptly triggered the release of small GdCuB, leading to the augmented penetration depth and the activation of MRI. The assembly switchable nanoclusters significantly improve the imaging resolution and photothermal therapy efficacy of breast cancer. To sum up, the strategy of constructing extracellular ATP-activated protein cluster dramatically ameliorated the theranostic performance of PBTA and could inspired the design of the next-generation PBTA. Furthermore, the successful of ATP-triggered release of negatively charged BSA nanoparticles also encouraged us to further exploit this strategy for the delivery of other small sized nanoparticles decorated with negatively charged surface, such as Fe₃O₄, quantum dots, gold nanoparticles and etc.

4. Experimental Section

**Materials:** Dextrin (Mₚ: 10 kDa), N,N’-Carbonyldiimidazole (CDI), 4-carboxyphenylboronic acid, EDC-HCl, NHS, copper(II) chloride dihydrate (CuCl₂·2H₂O), gadolinium(III) chloride hexahydrate (GdCl₃·6H₂O), and sodium sulfide nonahydrate (Na₂S·9H₂O) were purchased from Aladdin chemical regent company (Shanghai, China). RPMI-1640 medium, bovine serum albumin, Hoechst33258, and MTT were obtained from KeyGEN BioTECH (Nanjing, China).

**Synthesis of Dex–EDA:** Dextrin (2 mmol, 410 mg) was dissolved in DW and the pH was adjusted to 5.5 by adding 1 M NaOH. Subsequently, the carboxy of PBA was activated by EDC (98 or 195 mg) and NHS (57.5 or 115 mg) for 1 h under vigorous stirring. Then, the Dex–EDA solution (200 mg in 10 mL DW) was dropwise added to the PBA solution and the pH was adjusted to 5.5–6 by 1 M HCl. The reaction was protected by nitrogen. After 0.5 h, 10 mmol ethylenediamine was quickly added to the reaction solution and stirred under 45 °C water bath for 24 h. Finally, the excess CDI and EDA was removed by dialysis against DW for 48 h. The product was obtained by lyophilization. The chemical structures of Dex–EDA were characterized by ¹H-NMR and FTIR.

**Synthesis of Dex–EDA–PBA:** Phenylboronic acid functional Dex–EDA was synthesized by amidation reaction. Briefly, 4-carboxyphenylboronic acid (43 or 85 mg) was dissolved in DW and the pH was adjusted to 5.5 by adding 1 M NaOH. Subsequently, the carboxy of PBA was activated by EDC (98 or 195 mg) and NHS (57.5 or 115 mg) for 1 h under vigorous stirring. Then, the Dex–EDA solution (200 mg in 10 mL DW) was dropwise added to the PBA solution and the pH was adjusted to 5.5–6 by 1 M HCl. It was reacted at RT for 24 h. Finally, the product was obtained by dialysis and lyophilization. The chemical structures of Dex–EDA–PBA were characterized by ¹H-NMR and FTIR.

**Preparation and Characterization of GdCuB Nanoparticles:** The preparation of GdCuB nanoparticles was according to a biomineralization method. 34.096 mg CuCl₂·2H₂O, 73.36 mg GdCl₃·6H₂O and 1 g BSA was dissolved in DW separately. Briefly, CuCl₂·2H₂O and GdCl₃·6H₂O solution
were added to a one-neck flask and were stirred under 37 °C water bath. Then, the BSA solution was added to the mixture solution, a light green turbidity appeared. After reaction for 5 min, 2 mL 1% NaOH was added to adjust the pH to 12. The solution turn to transparent deep blue. Then 1.6 mL of 242.16 mg mL⁻¹ Na₂S₉H₄O was added to the mixture. The solution turned to deep brown, followed by reaction at 90 °C for 1 h. Finally, the product was processed with dialysis against DW for 24 h to remove the excess ions. A dark green cotton-like powder was obtained after lyophilization. The content of Gd and Cu was measured by ICP-MS.

For FITC labeled GdCuB (GdCuB-FITC) synthesis, 100 mg GdCuB was dissolved in Na₂CO₃/NaHCO₃ buffer (pH = 9). Subsequently, 10 mg FITC (dissolved in 200 µL DMSO) was added to the GdCuB solution. It was reacted for 12 h in the dark environment. After reaction, the solution was processed with dialysis against DW for 12 h, followed by lyophilization. The obtained GdCuB-FITC was stored at 4 °C in the dark.

Preparation and Characterization of DEP/GdCuB Nanocluster: For DEP/GdCuB nanocluster preparation, GdCuB solution was added to DEP (dissolved in pH 7.4 HEPES) solution and vortexed for 30 s. The nanocluster was incubated in 37 °C water bath for 30 min before use. The formation of nanocluster was checked by measuring the particle size, zeta potential, fluorescence quenching and TEM. The formulation was optimized by detecting the particle size and zeta potential of nanoclusters at various DEP/GdCuB w/v ratios (0.1, 0.2 0.5, 1, 2 and 4). DEP was dropwise added to the GdCuB-FITC to evaluate the fluorescence quenching of GdCuB-FITC. The weight ratios of DEP/GdCuB-FITC were among 0–1. The UV–vis spectrum of GdCuB and DEP/GdCuB was measured by ultraviolet–visible spectrophotometer. The serum stability of the nanoclusters was evaluated by incubating the clusters with FBS at 1:1 volume ratio and the dynamic size was monitored at 0.5, 1, 2, 4, 8, 12, 24, and 48 h.

ATP-Triggered Charge Reversal of DEP and Release of GdCuB Nanoparticles: The cationic dextrin–EDA was modified with different concentrations of DEP0, DEP1, and DEP2 represented the degree of substitution of PBA were 0%, 13.6%, and 29.7%, respectively. The ratios of PBA were calculated using the following equation:

\[ f_0 = \frac{1}{I} + \frac{x}{I_0} - \frac{x}{I_0} \times \frac{100}{I} + \frac{x}{I_0} \times \frac{100}{I} - \frac{x}{I_0} \]

where \( f_0 \) is the fluorescence intensity of free GdCuB-FITC without any polymer complexity, \( I_0 \) is the initial fluorescence intensity of GdCuB-FITC in the DEP/GdCuB cluster, and \( x \) is the percentage of released GdCuB-FITC. For ATP-triggered charge reversal study, ATP was increasingly added to DEP solution and the zeta potential was monitored by Malvern Mastersizer. The fluorescence resonance energy transfer (FRET) technology was applied to evaluate the ATP-triggered disassembly of GdCuB small nanoparticles. GdCuB was labeled with FITC and acted as the donor of the FRET pair. DEP was labeled with Rhb and acted as the receptor. The excitation wavelength of the detection was 488 nm and emission wavelength were among 500–700 nm. In addition, the FL images of Cy7 containing nanoparticles were prepared in 1.5 mL tubes. The DEP/GdCuB with or without 1 x 10⁻¹⁴ M ATP treatment was also compared here. The T₁-weighted and T₂-weighted images were recorded and the T₁ and T₂ were calculated.

ATP-Activated Tumor Deep Penetration: The in vivo ATP activated deep penetration of DEP/GdCuB was evaluated on 3D cell model. The 3D multicellular tumor spheroids was constructed as reported. Briefly, the 96-well plate was coated with 65 µL 2% agarose gel each well. 4T1 cells (200 cells per well in 200 µL medium) were seeded in the plate and grown for one week. The spheroid size reached 400 µm, the spheroids were transferred to the confocal dishes. The spheroids were incubated with DEP/GdCuB-FITC for 12 h in the presence or the absence of 1 × 10⁻⁴ M ATP. After incubation, the spheroids were washed with PBS and fixed by 4% paraformaldehyde. Finally, the images were obtained by CLSM in xy-stack with 10 µm intervals. For in vivo tumor deep penetration study, the GdCuB was labeled with FITC. The 4T1 tumor bearing mice was divided into 2 groups: GdCuB-FITC and DEP/GdCuB-FITC. The formulation was administrated with a dosage of 20 mg kg⁻¹ GdCuB-FITC (FITC: 1.5 mg kg⁻¹) by i.v. injection. The tumors were harvested at 24 h postinjection. Then, the isolated tumors were embedded in 4% paraformaldehyde, cut into 10 µm sections, and stained with DAPI. Blood vessels in the tumor frozen sections were visualized by CLSM using a CD31 antibody staining.

Animals: Female BALB/c mice (18–22 g) were bought from Yangzhou University. All the animal experiments were performed in compliance with the Guide for Care and Use of Laboratory Animals and were approved by China Pharmaceutical University.

In Vivo NIRF Imaging, Biodistribution Analysis and Blood Circulation Behavior: For NIRF imaging, GdCuB was labeled with near-infrared fluorescence probe (NIFP) Cy7. GdCuB-Cy7 and DEP/GdCuB-Cy7 was administrated by i.v. injection at Cy7 dosage of 2 mg kg⁻¹. The mice were observed and imaged by Caliper IVIS Lumina II in vivo image system at time intervals (12, 24, and 48 h). The biodistribution of GdCuB-Cy7 and DEP/GdCuB-Cy7 was determined by measuring the fluorescence intensity of Cy7 in the organs lysates. At 24 h postinjection, mice were sacrificed to collect various organs and tumor tissues, which were homogenized in the lysis buffer. The fluorescence intensity of Cy7 of each lysate was determined by the fluorescence spectrophotometer and calculated referred to the standard curve. For blood circulation behavior study, the tumor bearing mice were injected with GdCuB-Cy7 or DEP/GdCuB-Cy7 (n = 3). The blood samples were taken at 0.25, 0.5, 1, 2, 4, 8, 12, 24, and 48 h postinjection and the fluorescence intensity of Cy7 was measured by the fluorescence spectrophotometer.

In Vivo MR Imaging and Photothermal Imaging: The in vivo imaging study was carried on when the tumor size reached 200 mm³. For MR imaging, the mice were observed by a Bruker BioSpec 7T/20 cm...
system (Bruker, Ettlingen, Germany). The MR images were obtained before and after i.v. injection (6 and 24 h). The GdCuB nanoparticles and DEP/GdCuB nanoclusters were administrated at Gd\(^{3+}\) of 7.5 mg kg\(^{-1}\). The detailed MR imaging parameters were shown here: FOV = 2.4 cm, TR/TE = 662.4 ms/7.5 ms, slice = 12. For photothermal imaging, GdCuB and DEP/GdCuB were administrated by i.v. injection at Cu\(^{2+}\) dosage of 5 mg kg\(^{-1}\). The mice injected with PBS were set as control group. The NIR laser were irradiated at tumor site at 24 h postinjection. During irradiation, the temperature of tumor was monitored by an IR camera (Fluke VT04 Visual IR Thermometer, USA) at time intervals (0, 0.5, 1, 2, and 4 min). The images were processed by Fluke SmartView 4.3.

In Vivo Antitumor Efficacy and Biosafety Evaluation: The in vivo antitumor study was performed when the tumor size reached 150 mm\(^3\). Mice were randomly divided into 5 groups: 1) saline, 2) saline + laser (1 W cm\(^{-2}\), 1064 nm), 3) DEP/GdCuB, 4) GdCuB + laser, and 5) DEP/GdCuB + laser. The laser irradiation was carried on for 4 min per mouse at 24 h postinjection. The mice were treated every 4 days. Tumor size and body weight were recorded every 2 days. At day 16, the mice were sacrificed. Tumor and main organs were harvested from the body. The tumor was weighted and then immersed in formalin solution. All of the tissue samples were then embedded in paraffin, sliced and stained with H&E. For tumor apoptosis analysis, the nucleic of tumor cells were stained with DAPI and apoptosis cells were stained with dUTP-FITC for TUNEL analysis. To evaluate the biosafety of the nanoclusters, the main organs (heart, liver, spleen, lung, and kidney) were harvested, sliced and stained with H&E at day 16 after four treatments.

Statistical Analysis: All statistical analyses were performed using GraphPad Prism version 6.0 software. Data from the experiments were performed for three times or over three times. The results were expressed as the mean value ± standard deviation (mean ± SD). A two-tailed student’s t-test was performed for statistical analysis of the difference between the two groups. The p value of <0.05 was considered statistically significant between the data sets, where all significant values were indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

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

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Conflict of Interest
The authors declare no conflict of interest.

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