Research article

Yao Zhu, Yingying Liu, Zhongjian Xie, Tianzhen He, Lili Su, Fengjuan Guo, Gulzira Arkin, XiaoShu Lai, Jinfeng Xu* and Han Zhang*

Magnetic black phosphorus microbubbles for targeted tumor theranostics

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Abstract: Black phosphorus (BP) is attracting more and more interest for the biomedical application. The absorption in a wide spectral range and high photothermal conversion efficiency make BP suitable for photothermal therapy. However, BP alone is hard to realize the targeted therapy, which limits the precision and efficiency of the therapy. Magnetic microbubbles (MBs) are favored drug carriers because they can resist the shear force of blood flow in a magnetic field, which improves the efficiency of MBs adhesion to the vascular wall for targeted ultrasound diagnosis and therapy. This study first optimized the magnetic MBs configurations through controlling the connecting polyethylene glycol (PEG) chain length. The magnetic MBs with PEG2000 have been chosen for targeted BP nanosheets delivery due to the better stability and magnetic responsiveness. The magnetic black phosphorus microbubbles (MB_{BPM}) can realize the targeted tumor

theranostics *in vitro* and *in vivo*. They could be applied for the targeted ultrasound imaging with an enhanced echogenicity by three times when accumulated at the target site where the magnetic field is applied. As the NIR laser irradiation was applied on the accumulated MB_{BPM}, they dynamited and the temperature increased rapidly. It improved the cell membrane permeability, thus accelerating and enhancing a precision photothermal killing effect to the breast cancer cells, compared to BP alone.

Keywords: black phosphorus; magnetic microbubbles; photothermal therapy; targeted theranostics; US imaging.

1 Introduction

Cancer has become one of the major diseases that seriously endanger human health, thus many researchers have devoted to studying its mechanism, diagnosis, and therapy [1–4]. BP has a layered structure. It possesses a direct adjustable band gap from 0.3 to 2.0 eV as the thickness changes [5]. It has unique thermal, mechanical, and semiconductor properties, which has attracted wide attention of researchers for the application of thermoelectricity, energy storage, flexible electronics, and quantum information technology [6–9]. BP possesses ideal biodegradability and its potential in biomedical applications has been studied in drug delivery, photothermal therapy, photodynamic therapy, sonodynamic therapy, and photoacoustic imaging of diseases [10–20]. The BP alone cannot realize the targeted therapy and it is taken up by tumor cells mainly via endocytosis pathway, which takes about 4 h and limits the therapy efficiency [10, 21, 22]. Thus, the design of carriers for the targeted delivery of BP is important for its biomedical applications.

Ultrasound is considered a safe excitation source for various biomedical applications since it is noninvasive, nontoxic, and low-cost. Most importantly, ultrasound can achieve deeper tissue penetration than light [23–30]. In addition to its use in traditional ultrasonography applications, ultrasound is attracting increasing attention for

Yao Zhu and Yingying Liu contributed equally to this work.

*Corresponding authors: Jinfeng Xu, Department of Ultrasonography, Shenzhen Medical Ultrasound Engineering Center, Shenzhen People's Hospital, Second Clinical Medical College of Jinan University, First Clinical Medical College of Southern University of Science and Technology, Shenzhen 518020, China; and Han Zhang, Key Laboratory of Optoelectronic Devices and Systems of Ministry of Education and Guangdong Province, College of Physics and Optoelectronic Engineering, and Otolaryngology Department and Biobank of the First Affiliated Hospital, Shenzhen Second People's Hospital, Health Science Center, Shenzhen University, Shenzhen 518060, P.R. China, E-mail: jinfengxu@ext.jnu.edu.cn (J. Xu), hzhang@szu.edu.cn (H. Zhang). <https://orcid.org/0000-0002-9131-9767> (H. Zhang)

Yao Zhu, Yingying Liu, Tianzhen He, Lili Su, Fengjuan Guo, Gulzira Arkin and XiaoShu Lai, Department of Ultrasonography, Shenzhen Medical Ultrasound Engineering Center, Shenzhen People's Hospital, Second Clinical Medical College of Jinan University, First Clinical Medical College of Southern University of Science and Technology, Shenzhen 518020, China

Zhongjian Xie, Shenzhen International Institute for Biomedical Research, 518116 Shenzhen, Guangdong, China

disease therapy [31–44], similar to light-triggered therapy [45–48]. The resolution, sensitivity, and specificity of ultrasound diagnosis are significantly improved by ultrasound contrast agents. In recent decades, a diverse range of ultrasound contrast agents—including microbubbles (MBs), liposomes, and cerasomes—have been developed [49–62]. Among them, gas filled MBs have been approved by the US Food and Drug Administration (FDA) [63, 64]. Recently, MBs have been applied for targeted drug/gene delivery and controlled release based on ultrasound-targeted microbubble destruction (UTMD) [39, 40]. However, MBs have low binding ability, short retention time, and lack specificity and sensitivity, which limit their application in ultrasound theranostics [49–51, 54].

To achieve more efficient and specific binding ability, targeted MBs are created via adding targeting ligands such as folate, RGD peptide, and glycoprotein transferrin [65–71] to the microbubble shell surface. However, it only works at target areas with low blood flow speed, like microvasculature [72]. Comparatively, magnetic MBs can resist the shear force of blood flow and change their distribution in the blood vessel under a magnetic field. It can make the MBs closer to the endothelial cells of the vascular wall, thus to increase their contact probability with the target area and greatly improve the targeted binding efficiency. Thus, magnetic MBs possess great potential in the contrast-enhanced ultrasound imaging, targeted drug delivery and site directed vascular gene delivery [72–76]. If it is optimized, it will have broad clinical application prospects, such as improving sensitivity of visualizable treatment of cardiovascular diseases, enhancing the efficiency of diagnosis and therapies of tumors, and as imaging probes for immune adherence and other special biological processes. Different methods have been used to combine MBs and magnetic nanoparticles (NPs) to form magnetic MBs [73, 77–87]. The magnetic NPs have been embedded in the inner layer or shell of the MBs, and have also been attached to the shell surface of the MBs through certain chelating agents, biotin–avidin bridging, or electrostatic coupling. It affects the properties of the as synthesized magnetic MBs. Magnetic NPs embedded in the MB shell stiffen the surface and thus make the ultrasound image quality poorer [84, 85]. In contrast, coupling the magnetic NPs to the MB shell surface with polymer spacer arms, such as polyethylene glycol (PEG), increases the binding rate with minor effect on the surface properties [71]. The effects of PEG chain length and concentration on the mechanical property, dynamical properties, and stability of MBs have been investigated [72, 88]. However, the effects of the PEG chain length on the stability, the magnetic responsiveness, and the ultrasound image quality of the magnetic MBs have not been studied comprehensively.

In this study, different PEG chain lengths have been used to connect the magnetic NPs and the MBs, in order to find the chain length with these properties optimized. Then, the magnetic MBs with PEG2000 were applied for targeted BP nanosheets delivery, due to the better stability, magnetic responsiveness, and echogenicity. Compared with the BP alone, the magnetic black phosphorus MBs (MB_{BPM}) show accelerated and enhanced photothermal therapy efficiency. Moreover, it can achieve the integration of targeted disease diagnosis and treatment. The schematic diagram of the synthesis process of the MB_{BPM} and their application in the targeted theranostics are illustrated in Schematic 1. This work helps to design and optimize targeted theranostic magnetic MBs for enhanced diagnosis and therapy efficiency.

2 Results and discussion

2.1 Characterization of the BP nanosheets

The BP nanosheets were prepared by a liquid exfoliation method as previously reported [17]. Briefly, bulk BPs were firstly ground and then underwent probe sonication and bath sonication. From the transmission electron microscopy (TEM) image in Figure 1A, it can be seen that the BP nanosheets have a lateral size from 100 to 200 nm. The atomic force microscopy (AFM) in Figure 1B demonstrates that the BP nanosheets have a thickness of 3–3.5 nm, corresponding to 5–6 layers. The chemical element in the obtained BP nanosheets was investigated by X-ray photoelectron spectroscopy (XPS). Three XPS peaks were observed in Figure 1C, which located at 129.8, 130.6, and 133.9 eV, respectively. Among them, the peaks at 129.8 and 130.6 eV are assigned to P $2p_{3/2}$ and P $2p_{1/2}$ in BP while the peak at 133.9 eV corresponds to P in oxidized state (P ox) [89]. P ox peak is commonly observed in BP, because it is easily oxidized in open air [90]. Raman spectroscopy was applied to study the crystallinity of the bulk BPs and the exfoliated BP nanosheets (Figure 1D). In bulk BPs, three Raman peaks can be detected at about 362, 437, and 465 cm^{-1} , respectively. They are correspondingly consistent with the typical out-of-plane phonon mode A_g^1 and the in-plane phonon modes B_{2g} and A_g^2 [91]. The three phonon modes can also be seen in exfoliated BP nanosheets. It demonstrates that the obtained BP nanosheets are well crystallized but their Raman peak intensities dramatically decrease, which is attributed to the reduced number of layers [91]. Moreover, their A_g^2 vibration mode blue shifts compared to that in bulk BPs, also confirming the reduced thickness [92, 93]. The absorption of the exfoliated BP nanosheets was also measured. As shown in

Schematic 1: The schematic diagram of (A) the synthesis process of the MB_{BPM} and (B) its application for the targeted theranostics.

Figure 1E, the BP nanosheets have a broad absorption band covering the ultraviolet to infrared spectral range.

2.2 Properties of magnetic MBs controlled by PEG chain length

To optimize the magnetic MBs configuration for targeted delivery of the BP nanosheets, five types of magnetic MBs were synthesized by mixing the streptavidin-coated superparamagnetic Fe₃O₄ NPs with the biotinylated MBs with different connecting PEG chain lengths (none, PEG400, PEG1000, PEG2000, and PEG3400), as illustrated in Figure 2A. The concentrations of the as prepared biotinylated MBs are $\sim 1.3 \times 10^9$ MBs/mL. To verify the formation of the magnetic MBs, the magnet was used to see if they were magnetically responsive. It can be seen in the optical images (Figure 2B), that for both biotinylated and magnetic MBs, there is a cake layer in the upper portion of the solution but with different colors. It illustrates the existence of both MBs. When the magnet was placed near the MBs, biotinylated MBs showed no changes but magnetic MBs accumulated at the side where the magnet is placed (Figure 2C). It confirms the

magnetic MB formation. The morphology of the biotinylated MBs and magnetic MBs have been analyzed using scanning electron microscope (SEM) and transmission electron microscope (TEM) micrographs, respectively. As can be seen from Figure 2D, the MBs are spherical but they are collapsed under the electron beam with high energy, because they are filled with gas and the MBs shell are soft. The TEM micrograph of the magnetic MBs in Figure 2E further corroborates the attachment of the Fe₃O₄ NPs to the MB's surface.

An inverted fluorescence microscope was also used to study the morphology of the synthesized biotinylated MBs and magnetic MBs. The bright-field images of the five types of biotinylated MBs (no PEG spacer arm, MB_b; with PEG400, MB_{b400}; with PEG1000, MB_{b1k}; with PEG2000, MB_{b2k}; and with PEG3400, MB_{b3400}) and the corresponding magnetic MBs (MB_m, MB_{m400}, MB_{m1k}, MB_{m2k}, and MB_{m3400}) are shown in Figure 3A1–A5 and B1–B5, respectively. They are circle-shaped, which illustrates the MBs formation. The fluorescent images of the FITC-labeled magnetic MBs (Figure 3C1–C5) show green fluorescence along the hollow circle edge, which also confirms the coupling of the magnetic NPs to the MBs' shell surface.

To further comprehensively study the effect of the PEG chain length on the properties of the MBs, the particle size

Figure 1: (A)–(C) The transmission electron microscope (TEM) image, atomic force microscope (AFM) image, and X-ray photoelectron spectroscopy (XPS) spectrum of the exfoliated black phosphorus (BP) nanosheets, respectively. The scale bar in (a) is 0.2 μm . (B2) The height profile along the white line in (B1). (D) The Raman spectra of bulk BPs and BP nanosheets. (E) The absorption spectrum of the exfoliated BP nanosheets.

distribution and zeta potential of the biotinylated and magnetic MBs were measured. The binding capacity of the biotinylated MBs with the streptavidin-coated Fe_3O_4 NPs was then tested by measuring the percentage of magnetic MBs obtained.

For biotinylated MBs, their particle size distributions are $1.7 \pm 0.2 \mu\text{m}$ (Figure 4A) and their zeta potential are near -20 mV (Figure 4B). The PEG chain length shows negligible effects on them. After coupling the streptavidin-coated Fe_3O_4 NPs to the biotinylated MB shell surfaces, the average particle sizes of the MB_M , MB_{M400} , and MB_{M1k} were not different while the particle size distributions of MB_{M2k} and MB_{M3400} were changed to 2.7 ± 0.3 and $3.1 \pm 0.3 \mu\text{m}$, respectively (Figure 4A). The zeta potential of all the magnetic MBs remained negative but the absolute values decreased, compared with the biotinylated MBs (Figure 4B). The concentrations of the obtained magnetic MBs were also measured. The concentrations of MB_M and MB_{M400} were about $2.4 \times 10^8 \text{ MBs/mL}$ and that of MB_{M1k} was $6.4 \times 10^8 \text{ MBs/mL}$. The concentrations of MB_{M2k} and MB_{M3400} were 1.2×10^9 and $1.1 \times 10^9 \text{ MBs/mL}$, respectively. It was found that, MB_{b2k} and MB_{b3400} had significantly better binding capacity with streptavidin-coated Fe_3O_4 NPs than MB_b , MB_{b400} , and MB_{b1k} . It resulted in the markedly higher percentages of MB_{M2k} and

MB_{M3400} ($\sim 90\%$) than those of MB_M , MB_{M400} , and MB_{M1k} ($\sim 20\text{--}50\%$) in the original concentrations of the biotinylated MBs ($1.3 \times 10^9 \text{ MBs/mL}$) (Figure 4C).

The biotin linked to the MB's shell surface with shorter PEG chain length than PEG2000 may make it buried within the PEG2000 spacer arms added for long circulation *in vivo*. Thus, the streptavidin-coated Fe_3O_4 NPs are hindered in binding with the biotin [68, 94–96], which leads to the much lower magnetic MBs percentage. The coupled magnetic NPs to these MBs are shielded [95, 96], which results in the average particle sizes of MB_M , MB_{M400} , and MB_{M1k} similar to those of MB_b , MB_{b400} , and MB_{b1k} . In contrary, the biotin linked to the MB's shell surface with PEG2000 and PEG3400 is exposed, which can well connect with the streptavidin-coated Fe_3O_4 NPs and thus leads to much higher binding rate and the increased average particle sizes.

2.3 Stability of magnetic MBs controlled by PEG chain length

Owing to the intended practical application of the MBs, their stability was also tested (Figure 4D and E). A total of 20, 40,

Figure 2: (A) Illustration of the synthesized five types of biotinylated microbubbles (MBs) with different connecting polyethylene glycol (PEG) chain lengths (none, PEG400, PEG1000, PEG2000, and PEG3400) to combine with magnetic NPs for magnetic MBs formation. (B) Optical images of the as prepared biotinylated MBs and magnetic MBs without a magnet and (C) with a magnet placed near them. (D) The scanning electron microscope (SEM) micrograph of biotinylated MBs and (E) the TEM micrographs of magnetic MBs. The scale bar is 2 μm .

and 60 min after the storage of the biotinylated MBs and magnetic MBs in PBS (with the same initial concentration of 1×10^8 MBs/mL) in the ambient conditions, their concentrations were measured and compared with the as-prepared samples to evaluate the stability of each type of MBs. All of the biotinylated MBs spontaneously disassembled when stored in PBS in ambient conditions. MB_b, MB_{b400}, and MB_{b1k} disassembled gradually, with the remaining percentage being less than 80% after 60 min while MB_{b2k} and MB_{b3400} remained stable after 40 min with the remaining percentage being more than 80% after 60 min (Figure 4D). For the magnetic MBs, all of them decreased significantly with time when stored in PBS under ambient conditions (Figure 4E). Only ~30% of MB_M and MB_{M400} in the total MBs are left after 60 min. The percentage of MB_{M1k} is decreased to 60%. In comparison, the percentages of MB_{M2k} and MB_{M3400} were significantly higher than 70% even

after 60 min. In general, both biotinylated MBs and magnetic MBs with longer PEG chain lengths are more stable than those with shorter PEG chain lengths [84, 85].

2.4 Magnetic responsiveness of magnetic MBs controlled by PEG chain length

As the magnetic MBs are intended for efficient targeted theranostic applications, their magnetic responsiveness was measured with a home-made parallel plate flow chamber assay under shear forces from 5 to 45 dyn/cm², as shown in Figure 5A. The magnetic responsiveness of biotinylated MBs was measured as a control. Since the magnetic responsiveness of the biotinylated MBs with different PEG chain lengths show no significant difference (Figure 5B), only that of MB_b was shown in Figure 5C for a control.

Figure 3: (A1–A5) The bright-field images of biotinylated MBs with different PEG chain lengths (no PEG spacer arm, MB_b; with PEG400, MB_b400; with PEG1000, MB_b1k; with PEG2000, MB_b2k and with PEG3400, MB_b3400) and (B1–B5) the corresponding magnetic MBs (MB_M, MB_M400, MB_M1k, MB_M2k and MB_M3400). (C1–C5) The fluorescent images of FITC-labeled magnetic MBs with different PEG chain lengths. The scale bar is 5 μm .

It was shown in Figure 5C, as the shear force increased the number of captured magnetic MBs by the magnetic field decreased for all types of magnetic MBs studied. Compared with the biotinylated MBs, all of the magnetic MBs showed a higher captured percentage even under high shear forces of 45 dyn/cm^2 . It demonstrated that the magnetic MBs showed potential for targeted theranostic applications, even at target areas with a high blood flow speed, such as in the aorta. Among the magnetic MBs, MB_M2k, and MB_M3400 with longer PEG spacer arm length showed better magnetic responsiveness than MB_M, MB_M400, and MB_M1k. It may be because the higher magnetic NPs binding capacity to them compared to the MBs with shorter PEG length [95, 96].

2.5 Echogenicity of magnetic MBs controlled by PEG chain length

Achieving contrast-enhanced ultrasound images is one of the important intended functions of the MBs, therefore the effect of the PEG chain length on it was also studied. The locations of the ultrasound probe and the magnet placed near the agar plates are shown in Figure 6A. It showed no US

signal for PBS solution (Figure 6B). In contrast, the ultrasound signal in B-mode and contrast-mode using different types of biotinylated and magnetic MBs are significantly enhanced in Figure 6C.

For all of the biotinylated MBs, the ultrasound images showed no notable difference with and without magnet application. Although the MB_M, MB_M400, and MB_M1k can be attracted by the magnet in the parallel plate flow chamber assay, they showed no marked differences with and without a magnet under the ultrasound. It may be due to their poor magnetic responsiveness and stability. Moreover, in the parallel plate flow chamber, the distance between the magnetic MBs and the magnet is 0.13–0.17 mm, while it is about 1 cm, i.e. 80 times further in the echogenicity measurements. The magnetic field strength decreases with the distance, so the magnetic responsiveness of MB_M, MB_M400, and MB_M1k in the echogenicity measurements may be too low to accumulate at the target site. In contrast, when the magnet was applied, MB_M2k and MB_M3400 accumulated at the side where the magnet was placed and the ultrasound image signal was significantly enhanced. For quantitative analysis, the image signal in the contrast-mode at the accumulation region (region of

Figure 4: (A) The average particle size and (B) zeta potential of biotinylated MBs and the generated magnetic MBs with different PEG chain lengths; the black and red bars represent biotinylated MBs and magnetic MBs, respectively. (C) The percentage of magnetic MBs obtained in the total MB population. The remaining proportion of (D) biotinylated MBs and (E) magnetic MBs in the total MBs measured immediately after preparation and after storage in PBS under ambient conditions for 20, 40, and 60 min. Data represent mean with SD, $n = 5$. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Figure 5: (A) Schematic of the home-made parallel plate flow chamber assay. The captured percentage of (B) biotinylated MBs and (C) magnetic MBs by an applied magnetic field ($B \approx 1.2$ T) under shear forces from 5 to 45 dyn/cm². The biotinylated MBs were used as a control. Data represent mean $\pm$ SD, $n = 5$. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Figure 6: (A) Schematic diagram of the setup for acquiring ultrasonograms whilst applying a magnet. (B) The ultrasonogram of PBS solution. (C) B-mode and contrast-mode ultrasonograms of biotinylated and magnetic MBs suspensions in PBS without and with magnet application ($5 \times 10^6/\text{mL}$, pH 7.4, $B \approx 1.2$ T). (D) *In vitro* echogenicity at the region of interest (ROI) of MBs measured as signal enhancement normalized to the PBS solution signal. Data represent mean with SD, $n = 5$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

interest [ROI]) was recorded and the enhancement was normalized to the PBS solution signal (Figure 6B). It can be seen from Figure 6D, that for the biotinylated MBs and magnetic MBs with no magnet, the signal enhancement first increased and then decreased as the PEG chain length linking the biotin to the MB shell surface increased. $\text{MB}_{1\text{k}}$ and $\text{MB}_{1\text{k}}$ (no magnet) showed the highest echogenicity. Meanwhile, it was found that all of the magnetic MBs with no magnet showed a lower echogenicity compared with the biotinylated MBs. However, it was encouraging to find that the $\text{MB}_{2\text{k}}$ and MB_{3400} accumulated to the magnet side, leading to the echogenicity increased to three times and twice, respectively, which is much higher than those for all of the biotinylated MBs.

In both parallel plate flow chamber assay and echogenicity measurements, the magnetic responsiveness of magnetic MBs can be observed under magnetic field. It shows promising potential for the targeted diagnosis and drug delivery. The longer PEG chains linking the biotin to the MB shell surface and subsequently combined with the magnetic NPs were beneficial for improving the ultrasonogram quality as they accumulated under the magnetic field. In conclusion, the magnetic MBs with longer PEG spacer arms lengths (PEG2000 and PEG3400) showed increased average particle sizes compared with the corresponding biotinylated MBs. They also possessed better stability, magnetic responsiveness, and echogenicity than the magnetic MBs with shorter PEG chain lengths (none, PEG400, and PEG1000). These

effects are thought to be resulted from their different configurations. The biotin linked to the MBs' shell surface with PEG2000 and PEG3400 are well exposed and so is the coupled streptavidin-coated Fe_3O_4 NPs, which leads to the increased average particle size and good magnetic responsiveness. Contrarily, the biotin linked to the MBs' shell surface with no PEG, PEG400, or PEG1000 is buried [68, 94–96]. It is hard to couple with the streptavidin-coated Fe_3O_4 NPs, which make the magnetic MBs' average particle size with minor variation and the magnetic responsiveness worse. Thus, in the further study, the magnetic MBs with PEG2000 linking their surface with the magnetic NPs are chosen for the targeted delivery of BP nanosheets for the photothermal therapy.

2.6 Characterization of MB_{BPM}

To load the BP nanosheets on the surface of the magnetic MBs electrostatically, stearic-PEI 600 has been added in the MBs synthesis formula to achieve cationic MBs (MB_c). Its initial concentration was about 1.1×10^9 MBs/mL. BP nanosheets solution was added to the MB_c solution and they were incubated for 15 min. Then the magnetic NP dispersion was added to the mixture and incubated for further 15 min. The synthesized BP loaded magnetic MBs solution has a concentration of around 8.1×10^8 MBs/mL. The schematic of the finally obtained MB_{BPM} was shown in

Figure 7A. As shown in the photographs in Figure 7B, the white MB_c become black after adding BP nanosheets (i.e. MB_{BP}) and subsequently magnetic NPs (i.e. MB_{BPM}). The obtained MB_{BPM} can be accumulated to the side at which the magnetic field is applied. It illustrated the successful adsorption of BP nanosheets and magnetic NPs on the surface of the MBs. From the optical images in Figure 7C, MB_{BP} and MB_{BPM} have a black layer on the MBs surface. Their zeta potential has also been measured and they were presented in Figure 7D. The exfoliated BP nanosheets have an average zeta potential of -18.4 mV and the MB_c has a

surface charge of 20.5 mV. After mixing them, the zeta potential of MB_{BP} is negative. It further confirms the attachment of the BP nanosheets on the MB_c . The obtained MB_{BPM} possessed a surface charge of about -12.6 mV. The particle size distribution of bare MB_c is 2.7 ± 0.2 μm . The respective particle size distributions of MB_{BP} and MB_{BPM} are 3.6 ± 0.1 and 3.7 ± 0.3 μm , with the average particle sizes increased (Figure 7E).

To study the stability of the BP nanosheets loaded magnetic MBs, the remaining percentages of MB_{BP} and MB_{BPM} have been analyzed from as prepared to 1 h after the storage

Figure 7: (A) The schematic of the finally obtained magnetic black phosphorus microbubbles (MB_{BPM}). (B) The photographs and (C) the optical images of the cationic MBs (MB_c), black phosphorus microbubbles (MB_{BP}), and MB_{BPM} . The scale bars are all 20 μm (D) The zeta potential and (E) the average particle sizes of BP nanosheets, MB_c , MB_{BP} , and MB_{BPM} . (F) The remaining percentage of MB_{BP} and MB_{BPM} with time. (G) The absorption spectra of the subnanat in the MB_{BP} solution with time. (H) B-mode and contrast-mode ultrasonograms of the MB_{BPM} suspension in PBS without and with magnet application ($5 \times 10^6/\text{mL}$, pH 7.4, $B \approx 1.2$ T) and the ratio of the signal intensity at the target region (ROI 1) to that at the nontarget region (ROI 2). Data represent mean with SD, $n = 5$. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

in the ambient condition. It can be observed in Figure 7F, MB_{BP} is stable without obvious reduction. Moreover, through measuring the absorption of the supernatant in the MB_{BP} solution, it shows no detected BP nanosheets desorption from MB_{BP} (Figure 7G). It illustrates the BP nanosheets are stably electrostatically adsorbed on the MBs surface. MB_{BPM} has decreased with time, which is consistent with the previous study on the magnetic MBs but its percentage still remains more than 80% after 1 h.

The ultrasonograms of the final BP-incorporated magnetic MB without and with the magnet application have been measured, which is shown in Figure 7H. To verify its targeting effect, the signal intensity of the target region where the magnet is placed (ROI 1) is divided by that of nontarget region (ROI 2). It is enhanced significantly from about 1 as no magnet is applied to about 4 and 7 as the magnet is applied for B-mode and contrast mode, respectively. It demonstrates a good targeting effect of the MB_{BPM} .

2.7 Photothermal effect of MB_{BPM}

The BP nanosheets have a wide absorption spectrum from ultraviolet to infrared spectral range (Figure 1E), which shows a promising potential in photothermal therapy under near infrared (NIR) light. To study the photothermal performance of the synthesized MB_{BPM} , the MB_{BPM} dispersion underwent magnetic field for 3 min to attract the MB_{BPM} to the bottom of the eppendorf (EP) tube and then it was irradiated with an 808 nm laser at 2.0 W/cm^2 for 10 min. The measurement

schematic is shown in Figure 8A. For comparison, the photothermal performance of BP nanosheets and the PBS were also measured in the same condition. As can be seen in Figure 8B–D, without NIR laser irradiation, the temperature almost remains at about 28°C for all of the PBS, BP nanosheets, and MB_{BPM} solutions. Under NIR laser irradiation for 10 min, the temperature of the BP nanosheets and MB_{BPM} dispersion could increase to more than 40°C , but the different regions could reach different temperatures. For BP nanosheets dispersion, the temperature gradually increased from the top (region 2) to the bottom (region 1) with the irradiation time increasing (Figure 8D). The temperature in region 2 reached about 44°C while that in region 1 reached just about 36°C . On the contrary, the MB_{BPM} was attracted at the bottom (region 1), the temperature increased rapidly at the accumulation site to about 42°C , while that in the upper region 2 is lower and slower. The rate of the temperature increase and the finally reached temperature after 10 min of NIR irradiation are different in different regions for BP nanosheets alone and MB_{BPM} suspension under the magnetic field application. Figure 8A show the measurement schematic on the photothermal performance of the MB_{BPM} . Under the magnetic field, the MB_{BPM} accumulate at the bottom region 1, resulting in the local concentration of BP nanosheets in the region 1 high while that in the upper region 2 much lower. Therefore, the temperature in region 1 increases quickly to more than 40°C . Contrarily, the temperature increases much slower and the final temperature is lower in the region 2 for the MB_{BPM} . However, for BP nanosheets alone, they are still uniformly dispersed, which is not affected by the magnetic field and not

Figure 8: (A) The measurement schematic on the photothermal performance of the MB_{BPM} under a magnetic field. (B) The infrared (IR) thermal images of PBS, BP nanosheets, and MB_{BPM} without and with 808 nm laser irradiation (2.0 W/cm^2) for 10 min. (C) and (D) The corresponding photothermal heating curves of the PBS, BP nanosheets, and MB_{BPM} without and with the 808 nm laser irradiation (2.0 W/cm^2) for 10 min, respectively.

like the case of MB_{BPM} in Figure 8A. Since the upper region 2 is closer to the NIR laser than the bottom region 1, the temperature gradually increases from the region 2 to the region 1 with the irradiation time increasing.

2.8 *In vitro* photothermal therapy with MB_{BPM}

To study the targeted photothermal therapy effect of the MB_{BPM} *in vitro*, MB_{BPM} and MB_{BP} were incubated with the MCF-7 breast cancer cells under the magnetic field (about 1.2 T) for 3 min and then the 808 nm laser irradiation (2.0 W/cm^2) was applied for 10 min. It can be seen from Figure 9A,

there was no obvious MB_{BP} (optical image of the MCF-7 breast cancer cells incubated with MB_{BP}) but numerous MB_{BPM} (optical image of the MCF-7 breast cancer cells incubated with MB_{BPM} before NIR irradiation) can be observed on the MCF-7 cells. It illustrated the capacity of the magnetic MBs for targeted delivery of the BP nanosheets. The circle-shaped MB_{BPM} can be seen before the NIR irradiation (optical image of the MCF-7 breast cancer cells incubated with MB_{BPM} before NIR irradiation), but they diminish after the NIR irradiation (optical image of the MCF-7 breast cancer cells incubated with MB_{BPM} after NIR irradiation), which are dynamited. After the treatments with different concentrations of MB_{BPM} (from 1×10^5 to $1 \times 10^7 \text{ MB}_{BPM}/\text{mL}$, $5 \times 10^{-5} \mu\text{g BP nanosheets}/\text{MB}$), the cells were further cultured for 12 h. Afterwards, the MCF-7 cells

Figure 9: (A) The optical images of MCF-7 cancer cells incubated with MB_{BP} and MB_{BPM} under the magnetic field ($B \approx 1.2 \text{ T}$) for 3 min and then under the NIR laser irradiation. (B) The fluorescence images of the treated MCF-7 cancer cells stained with calcein AM (green) and PI (red) for live and dead cells, respectively. (C) The corresponding relative viability of the MCF-7 cancer cells treated with different concentration of MB_{BPM} under the 808 nm laser irradiation (2.0 W/cm^2) for 10 min. (D) The TEM image of the MCF-7 cancer cells incubated with MB_{BPM} ($1 \times 10^7 \text{ MB}_{BPM}/\text{mL}$) under the magnetic field ($B \approx 1.2 \text{ T}$) for 3 min and then treated under the 808 nm laser irradiation (2.0 W/cm^2) for 10 min. 1: the nanomaterials uptaken inside the MCF-7 cells indicated by the red arrows; 2 and 3: the mitochondrion already collapsed and with the inner membrane ridges irregularly arranged indicated by the green and blue arrows, respectively. 4: Autophagolysosome containing nanomaterials and some subcellular organelles indicated by the yellow arrows. The scale bar in the optical images of MCF-7 cancer cells incubation with MB_{BP} and MB_{BPM} before NIR irradiation in (A) is $400 \mu\text{m}$. Data represent mean with SD, $n = 5$. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

were rinsed with PBS three times and then costained with Calcein AM and PI for 30 min. After rinsing with PBS, they were observed by the inverted fluorescence microscope and the results were shown in Figure 9B. The number of live and dead cells were counted by ImageJ software and the cell viability was calculated (Figure 9C). The corresponding relative cell viability decreased from about 80% to no more than 10% as the MB_{BPM} concentration increased to $1 \times 10^7 \text{ MB}_{\text{BPM}}/\text{mL}$. The TEM analysis for the cellular uptakes of the MB_{BPM} ($1 \times 10^7 \text{ MB}_{\text{BPM}}/\text{mL}$) in MCF-7 cells was also performed, in order to assess the interactions of the MB_{BPM} with the cell membrane and the influence of the photothermal therapy on the cells. It can be observed from Figure 9D, as the red arrows indicate, a huge amount of nanomaterials including BP nanosheets and some iron oxide NPs have been uptaken inside the cells only after 3 min of incubation with the MCF-7 cells under the magnetic field and 10 min of treatment under the NIR irradiation. It demonstrates that the cell membrane

permeability is improved, thus shortening the uptake time of the BP nanosheets inside the cells greatly. Moreover, it also can be seen in the TEM image of the MCF-7 cells after the photothermal therapy, the mitochondrion has already collapsed or its inner membrane ridges are irregularly arranged, as the green and blue arrows indicate. These illustrate the photothermal therapy with the MB_{BPM} lead to the apoptosis of the MCF-7 cells and the generation of the autophagolysosome, as the yellow arrows indicate.

The optimized concentration, i.e. $1 \times 10^7 \text{ MB}_{\text{BPM}}/\text{mL}$ was used in the further study of the *in vitro* photothermal therapy effect comparison between the MB_{BPM} and the BP nanosheets (Figure 10). It was found that, BP nanosheets and MB_{BPM} addition without NIR irradiation or only NIR irradiation did not kill the MCF-7 cells (Figure 10A). With the magnetic field applied, incubation of BP nanosheets with the MCF-7 cells for 3 min and then applying 808 nm laser of $2.0 \text{ W}/\text{cm}^2$ for 10 min did not lead to obvious killing effect. Extending the

Figure 10: (A) The fluorescence images of the MCF-7 cancer cells treated with BP nanosheets, MB_{BPM} , or none of them (blank) without or with NIR laser irradiation after incubation for 3 min, 3 h, or 5 h and then stained with calcein AM (green) and PI (red) for live and dead cells, respectively. (B) The corresponding relative viability of the treated MCF-7 cancer cells. The scale bar is $400 \mu\text{m}$. Data represent mean with SD, $n = 5$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

incubation time to 3 h, the viability decreased to less than 50% (Figure 10B). It may be due to that the BP nanomaterials taken up by tumor cells via endocytosis pathway takes about 4 h [10, 21, 22]. In contrast, incubation of MB_{BPM} with the MCF-7 cells under the magnetic field for 3 min and then applying the NIR laser irradiation almost kill the MCF-7 cells completely. Extending the incubation time to 3 h showed no obvious difference. To further extend the incubation to 5 h, the cell viabilities of the treated MCF-7 cells show no obvious difference after the treatment with both the BP nanosheets alone and the MB_{BPM}. It is proposed that the accelerated and enhanced photothermal killing effect was attributed to: 1. the targeted effect of the MB_{BPM}, resulting in the large amount of MB_{BPM} accumulated at the breast cancer cells and the temperature rapidly increase locally; 2. the dynamited MB_{BPM} under the NIR laser irradiation (Figure 9A) is beneficial for increasing the cell membrane permeability and thus the BP nanosheets could be delivered into the MCF-7 cells quickly [97]; 3. The local mild temperature increase also improved the cell membrane permeability [98]. Thus, MB_{BPM} accompanied with the magnetic field and NIR laser irradiation can accelerate and enhance the photothermal therapy effect.

2.9 *In vivo* ultrasound imaging with MB_{BPM}

To show the potential of the MB_{BPM} for the targeted diagnosis, the ultrasound images of the tumors before injection, after injection of 20 min and after the burst of MB_c and MB_{BPM} have been obtained (Figure 11A). It can be seen that,

after the injection of both MB_c and MB_{BPM}, the ultrasound signals at the tumors were enhanced, but which is higher for MB_{BPM}. For a quantitative analysis, the echo-power-time profile of the tumor regions (ROI) 15 s before and after the burst of MB_c and MB_{BPM} after 20 min of their injection for their accumulation under the magnetic field. As shown in Figure 11B, the US signal intensity is higher for MB_{BPM} before their burst, which illustrated its better magnetic responsiveness than MB_c. Since the US signal before the burst of the MBs comes from both the adherent MBs and the US signal after the burst of the MBs comes only from the circulating MBs [99], the signal intensity before the burst minus that after the burst (i.e. the differential targeted enhancement parameter, dTE) was used to evaluate their targeting efficiency. In Figure 11C, the dTE parameter was shown for MB_c and MB_{BPM} and it was much bigger of MB_{BPM} (12.1 ± 1.2 a.u.) than that of MB_c (3.8 ± 0.6 a.u.), which demonstrated the much higher targeting efficiency.

2.10 *In vivo* photothermal therapy with MB_{BPM}

To show the targeted PTT efficiency of the MB_{BPM}, the tumor-bearing mice were injected intravenously the PBS, BP nanosheets, and MB_{BPM}, with the magnet placed near the tumor sites. After 10 min of the injection, the tumors were irradiated by the 808 nm laser (2.0 W/cm^2 , 10 min). The treatment was conducted every three days for three times.

Figure 11: (A) Ultrasound images of the tumors before injection, after injection for 20 min and after the burst of MB_c and MB_{BPM}. (B) The echo-power-time profile of the tumor regions (ROI) 15 s before and after the burst of MB_c (blue dots) and MB_{BPM} (grey dots) and their average values (MB_c: orange dots and MB_{BPM}: purple dots). (C) The differential targeted enhancement (dTE) parameter for the MB_c and MB_{BPM}. Data represent mean with SD, $n = 5$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 12:

The photographs of the tumor-bearing mice and the isolated tumors on the 19th day after the photothermal therapy have been captured. As shown in Figure 12A, the tumor volume was the biggest after the PTT with PBS. Compared with that, the tumor volume was smaller after the PTT with BP nanosheets and that was the smallest after the PTT with MB_{BPM}. From the H&E staining of the tumor tissue slices with the PTT in the three groups, it can be observed that the tumor cells mostly maintained their normal morphology while those were destroyed and became necrotic for the BP nanosheets and MB_{BPM} groups. Moreover, the tumor cells were more severely destroyed. It demonstrated that the MB_{BPM} has higher *in vivo* PTT efficiency. The tumor volumes and body weights of the tumor-bearing mice received the PTT with PBS, BP nanosheets, and MB_{BPM} were also measured every other day. It illustrated that the tumor grew fast during 19 days after the PTT with PBS (Figure 12B). In comparison, the tumor grew slower after the PTT with BP nanosheets while the tumor growth was almost completely suppressed from the 7th day, confirming the higher efficient PTT of MB_{BPM}. The weight of the treated mice was nearly not affected by the treatments (Figure 12C). It was also found that the no significant histological abnormalities were seen from the H&E staining of the major organs after the PTT with MB_{BPM} (Figure 12D), which demonstrated its good biosafety.

3 Conclusions

The few-layer BP nanosheets of 100–200 nm have been synthesized via liquid phase exfoliation. It possesses a wide absorption band even in the NIR range. To deliver them into cancer cells for targeted theranostics, different configurations of magnetic MBs were achieved via the combination of streptavidin-coated Fe₃O₄ NPs and biotinylated MBs, with different connecting PEG chain lengths (no PEG spacer arm, PEG400, PEG1000, PEG2000, and PEG3400). As the PEG chain length increased, the stability was improved. For the shorter connecting PEG chain lengths, the biotin is shielded and thus the streptavidin-coated Fe₃O₄ NPs is hindered to couple with it. It results in the low yield of MB_M, MB_{M400}, and MB_{M1k} (lower than 50%) and poor magnetic responsiveness. In contrast, for the magnetic MBs with longer connecting PEG chain lengths—MB_{M2k} and MB_{M3400}—the percentages were near 100%. Moreover, MB_{M2k} and MB_{M3400} showed better stability, magnetic responsiveness, and echogenicity under magnetic field than the other magnetic MBs. Then, a precision delivery strategy by magnetic MBs with PEG2000 was adopted for targeted BP nanosheets delivery. The synthesized MB_{BPM} are stable and can achieve targeted theranostics. They accumulate at the site with magnetic field application, increasing the US imaging signal intensity

significantly both *in vitro* and *in vivo* and also leading to the local temperature increases rapidly to a saturation. Moreover, the *in vitro* and *in vivo* photothermal therapy also demonstrated that the MB_{BPM} can dramatically improve the photothermal therapy efficiency with a significantly shortened incubation time needed for BP nanosheets delivered into the cancer cells under the magnetic field and NIR laser irradiation.

4 Methods

4.1 Materials

The main component materials of the lipid microbubbles—1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC)—were purchased from Avanti Polar Lipids (Alabaster, USA). The coupling agent with different PEG chain lengths, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[biotinyl(polyethylene glycol)-*x*] (DSPE-PEG_x-biotin) with *x* = 0, 400, 1000, 2000 and 3400, were obtained from Ruixi Biological Technology Co. Ltd (Xi'an, China). The streptavidin-coated superparamagnetic Fe₃O₄ NPs (5 mg/mL, ~130 nm) were purchased from Zhongkeleiming Technology Co. Ltd (Beijing, China). The bulk BP was obtained from the supplier of Smart-Elements (Austria) and anhydrous *N*-methyl-2-pyrrolidone (NMP) (99.5%) was bought from Aladdin Bio-Chem Technology Co. LTD (Shanghai, China). Calcein AM and PI were obtained from KeyGEN BioTECH (Nanjing, China). All of the materials were used as received without further modification.

4.2 Preparation of magnetic MBs

First, the biotinylated MBs were synthesized. DSPC, DSPE-PEG2000, and DSPE-PEG_x-Biotin (*x* = 0, 400, 1000, 2000, and 3400) were dissolved in a mixture of 18 mL of chloroform and 2 mL of methanol with a molar ratio of 82:9:9, as previously reported [79, 81]. The mixture was then magnetically stirred for 30 min. After rotary evaporation, vacuum drying, hydration and ultrasound treatment, the obtained solution was evacuated and octafluoropropane (C₃F₈) was introduced. Finally, the MBs were achieved with shaking for 30 s. MBs were purified by two centrifugation (400 g)/redispersion cycles. The as synthesized biotinylated MBs with different PEG chain lengths were denoted MB_b, MB_{b400}, MB_{b1k}, MB_{b2k}, and MB_{b3400}, for *x* = 0, 400, 1000, 2000, and 3400, respectively.

To prepare the magnetic MBs, a suspension of streptavidin-coated superparamagnetic Fe₃O₄ NPs was added to the five as synthesized biotinylated MB solutions for 15 min. To reduce the possibility that one streptavidin binds multiple biotins, and thus avoid the aggregation of the magnetic MBs, the streptavidin-coated superparamagnetic Fe₃O₄ NPs firstly undergo ultrasound treatment and its ratio to biotinylated MBs was set to 4:1. Then the obtained magnetic MB solutions were subjected to two centrifugation (400 g)/redispersion cycles to remove the magnetic NPs not coupled to the MB shell surface. Finally, a magnet (25 × 10 × 5 mm, ~1.2 T) was used to attract the magnetic MBs while the nonmagnetic MBs in the solutions were collected for measuring the nonmagnetic MBs. The magnet was then removed and the purified magnetic MBs were

redispersed in PBS for further characterization. The obtained magnetic MBs were denoted MB_m, MB_{m400}, MB_{m1k}, MB_{m2k}, and MB_{m3400}, for *x* = 0, 400, 1000, 2000, and 3400, respectively. To check the magnetic NPs connected on the MBs surface, the Labeling Check Reagent-FITC (MACS Miltenyi Biotec, Germany) was added in the magnetic MBs suspension for 15 min at 4 °C and the excess Labeling Check Reagent-FITC was removed by two centrifugation (400g)/redispersion cycles.

4.3 Preparation of MB_{BPM}

The BP nanosheets were synthesized via liquid exfoliation as previously reported [89]. An amount of 20 mg of the bulk BP powder dispersed in 2 mL of NMP (10 mL) was ground. Then 18 mL additional NMP was added to the dispersion and the mixture underwent probe sonication for 8 h (duty cycle of 50%) using a power of 260 W. Afterwards, the mixture underwent sonication overnight with a power of 300 W. To avoid the overheat, they were performed in an ice bath. The dispersion was centrifuged at 7000 rpm for 20 min and the collected supernatant was further centrifuged at 15,000 rpm for 5 min. Some of the obtained precipitate was dispersed in PBS for the measurement and the others was dispersed in NMP and stored at 4 °C.

For the electrostatic adsorption of BP nanosheets on the MBs surface, the stearic-PEI600 was added in the MBs formulation to synthesize MB_c with the molar ratio of DSPC, DSPE-PEG2000, DSPE-PEG2000-biotin, and stearic-PEI600 to be 46:9:9:36. Then the BP nanosheets dispersion was mixed with the MB_c. After their incubation for 15 min, the magnetic NP dispersion was then added to the mixture and incubated for further 15 min. To mix them well, the mixture was shaken gently. Finally, the mixture was centrifuged at 1400 rpm for 4 min and the unadsorbed BP and nonconnected magnetic NPs in the suspension was removed.

4.4 Property characterization

The particle size distribution and zeta potential of all of the biotinylated, magnetic MBs, and MB_{BPM} were analyzed by dynamic light scattering (DLS) with a Zetasizer Nano ZSE (Malvern, United Kingdom). Since the zeta potential of samples was influenced by PBS, it was measured in deionized water. The concentration of the as prepared MBs was measured using accuser (Particle Sizing Systems, PSS A7000AD, USA). The binding rate of the magnetic NPs to the MBs was evaluated from the percentage of magnetic MBs in the entire MB population, which could be calculated using the formula: (entire MBs concentration – nonmagnetic MBs concentration)/entire MBs concentration. Their morphologies were observed with an inverted fluorescence microscope (Leica DMI8, Germany), scanning electron microscopy (SEM, ZEISS SUPRA55, Germany) and transmission electron microscopy (TEM, Hitachi 7500, Japan). The thickness of BP nanosheets was measured using Atomic Force Microscope (AFM, Bruker, Germany). XPS was achieved via a ULVAC PHI 5000 Versa Probe II (Japan). Raman spectra was obtained by Witec Alpha 300R using a 532 nm continuous-wave laser (Witec, Germany). Absorption spectra were acquired through a Tecan Spark multifunctional microplate reader (Switzerland).

4.5 Stability

To test the stability of the biotinylated MBs, magnetic MBs, BP MB_{BP}, and MB_{BPM}, they were dispersed in PBS solution and then their

remaining percentage were measured as prepared and after storage in ambient conditions for 20, 40, and 60 min.

4.6 Magnetic responsiveness

Analysis of the magnetic responsiveness was conducted with a home-made parallel plate flow chamber assay. A total of 1 mL of magnetic MBs (with the same initial concentration of 1×10^8 MBs/mL) was injected into a vacuum parallel plate flow chamber (Glycotech 31-001, USA) by a stepping motor (Yuhui, China) through capillary tubing ($r = 0.15$ cm) at shear forces of 5–45 dyn/cm². A magnet ($25 \times 10 \times 5$ mm, ~ 1.2 T) was placed on the chamber throughout the experiments to capture the magnetic MBs, and then they were redispersed in 1 mL of PBS. The magnetic responsiveness was evaluated from the ratio of captured MBs to MBs originally injected and was plotted as a function of the shear force.

4.7 *In vitro* echogenicity

To obtain the *in vitro* ultrasound imaging, the Vevo 2100 ultrasound imaging platform (VisualSonics, Canada) was used. Agarose gel powder and microcentrifuge tube were used to prepare agar plates with holes, and MBs of the same concentration (5×10^6 MBs/mL, 400 μ L) were added to the agar plates. Their ultrasound images and the signal intensities in the region of interest (ROI) were recorded (B-mode and contrast mode, frequency of 18 MHz, power of 2%, gain of 35 dB) under the magnet for 20 min. The signal enhancement was normalized by that of PBS solution of the same volume to evaluate the *in vitro* echogenicity.

4.8 Photothermal performance

0.5 mL of samples in EP tubes were irradiated by a fiber-coupled continuous laser of 808 nm (Yuanming Laser Technology, China) with a power density of 2.0 W/cm² for 10 min. Real-time thermal imaging was captured and the temperature was recorded by the infrared thermal imaging camera (Fluke Ti27, USA).

4.9 *In vitro* photothermal therapy study

MCF-7 cells (human breast cancer cells) were seeded in a 96-well plate for 12 h with 200 μ L of DMEM (HyClone) supplemented with 10% (volume ratio) of fetal bovine serum and kept in an incubator consisting of 5% CO₂ at 37 °C. Then they were incubated with MB_{BPM} or BP nanosheets for 3 min or 3 h under magnetic field and then were irradiated by 808 nm laser with the power of 2.0 W/cm² for 10 min. For each sample, three multiple holes were set. The height of laser tip to 96-well plates was adjusted to cover one well. After the treatments, the cells were further cultured for 12 h. Afterwards, the MCF-7 cells were rinsed with PBS three times and then costained with Calcein AM and PI for 30 min. After rinsing with PBS, they were observed by the inverted fluorescence microscope. The relative cell viability was calculated with ImageJ software depending on the fluorescence images. After the treatment with MB_{BPM}, the cells were washed with PBS to remove the materials. Then they were detached, centrifuged, fixed, and dehydrated. The cell pellets were infiltrated in a mixture of epoxy resin in 100% ethanol and leave it polymerized to make ultrathin slices.

Finally, their TEM images were captured using the 120 kV transmission electron microscope (Tecnai G2 Spirit BioTWIN, FEI, USA).

4.10 Establishment of subcutaneous tumor model

The Female BALB/c nude mice aged six weeks old with the weight about 20 g were bought from the Huaifukang Biotechnology Co., Ltd. (Beijing, China). To establish the subcutaneous tumor model, MCF-7 cells (5×10^7 /mL, 100 μ L) were injected subcutaneously into their right forelimb armpit. When the tumor diameters reached approximately 5–7 mm, the nude mice were used for the *in vivo* ultrasound imaging and the *in vivo* photothermal therapy.

4.11 *In vivo* ultrasound imaging

The tumor-bearing nude mice were first anesthetized with 0.5% pentobarbital (30 mg/kg mouse weight). The Vevo 2100 ultrasound imaging platform (VisualSonics, Canada) was used to obtain the contrast-enhanced ultrasound imaging of the tumors before the MBs injection. Then they were administrated intravenously the MB_c and the MB_{BPM} ($n = 5$ for each group, 1×10^7 MBs/mL, 150 μ L), with the magnet placed next to the tumors. After 20 min of their injection for the MBs accumulation, the US imaging was captured for 15 s, followed by the burst of the MBs and the magnet removed. The US imaging is captured for 15 s further to allow freely circulating MBs to replenish in tumors. The differential targeted enhancement (dTE) parameter was analyzed with the built-in Vevo CQ software to evaluate the targeting effect of the MB_{BPM}.

4.12 *In vivo* photothermal therapy study

To study the PTT efficacy of the MB_{BPM} *in vivo*, the tumor-mice were administrated intravenously the PBS, MB_{BP}, and the MB_{BPM} ($n = 5$ for each group, 150 μ L) with the magnet placed next to the tumors. After 10 min of the injection, the tumors were irradiated by the 808 nm laser (2.0 W/cm², 10 min). The treatment was conducted every three days for three times and the changes in the tumor volume and body weight were recorded for 19 days. The tumor size was measured using a vernier caliper, and the volumes were calculated by the equation (volume = length $\times$ width²/2). At the 19th day, all of the mice were executed cervical dislocation and the tumors of each group were isolated for H&E staining. To assess the biosafety of the MB_{BPM}, the organs of heart, liver, spleen, lung, and kidney of the MB_{BPM} group were also isolated for H&E staining.

4.13 Statistical analysis

Comparisons between two groups of data were made using the unpaired *t*-test. The means of more than two groups were compared with the one-way ANOVA or two-way ANOVA, followed by post Tukey's pairwise comparisons. Probability values $p < 0.05$ were considered statistically significant. Statistical analyses were carried out with Prism software packages (version 5.01).

Author contributions: Y.Z. performed all the characterizations and wrote the manuscript. Y.-Y.L. designed the experiments. Z.-J.X. edited and revised the manuscript. T.-Z.H.

synthesized microbubbles and L.-L.S. conducted echogenicity measurements. F.-J.G. prepared magnetic microbubbles. G.A. cultured cells and performed photothermal measurements. X.-S.L. performed TEM measurements. J.-F.X. and H. Z. conceived and supervised the study. All authors approved the manuscript.

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