Research article

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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 sheer 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 (MBBPM) 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
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 sheer 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 (MBBP) 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 MBBP 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 2p1/2 and P 2p3/2 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 \( \Gamma_1 \) and the in-plane phonon modes \( \Gamma_2 \) and \( \Gamma_3 \) [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 \( \Gamma_1 \) 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
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, MBb; with PEG400, MBb400; with PEG1000, MBb1k; with PEG2000, MBb2k and with PEG3400, MBb3400) and the corresponding magnetic MBs (MBM, MBM400, MBM1k, MBM2k, and MBM3400) 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
distribution and zeta potential of the biotinylated and magnetic MBs were measured. The binding capacity of the biotinylated MBs with the streptavidin-coated Fe₃O₄ NPs was then tested by measuring the percentage of magnetic MBs obtained.

For biotinylated MBs, their particle size distributions are 1.7 ± 0.2 μm (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 Fe₃O₄ NPs to the biotinylated MB shell surfaces, the average particle sizes of the MBM, MB₄₀₀, and MB₁₀₀₀ were not different while the particle size distributions of MB₂₀₀ and MB₃₄₀₀ were changed to 2.7 ± 0.3 and 3.1 ± 0.3 μm, 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 MB₅₀ and MB₅₀₀₀ were about 2.4 × 10⁸ MBs/mL and that of MB₁₀₀₀ was 6.4 × 10⁷ MBs/mL. The concentrations of MB₅₀₀ and MB₃₄₀₀ were 1.2 × 10⁹ and 1.1 × 10⁹ MBs/mL, respectively. It was found that, MB₂₀₀ and MB₃₄₀₀ had significantly better binding capacity with streptavidin-coated Fe₃O₄ NPs than MB₅₀, MB₅₀₀₀, and MB₁₀₀₀. It resulted in the markedly higher percentages of MB₂₀₀ and MB₃₄₀₀ (~90%) than those of MB₅₀, MB₅₀₀₀, and MB₁₀₀₀ (~20–50%) in the original concentrations of the biotinylated MBs (1.3 × 10⁹ 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 Fe₃O₄ 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 MB₅₀, MB₅₀₀₀, and MB₁₀₀₀ similar to those of MB₂₀₀, MB₃₄₀₀, and MB₁₀₀₀. 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 Fe₃O₄ 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,
and 60 min after the storage of the biotinylated MBs and magnetic MBs in PBS (with the same initial concentration of $1 \times 10^8$ 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 $\sim 30\%$ 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/cm$^2$, 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.
It was shown in Figure 5C, as the sheer 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 sheer forces of 45 dyn/cm². 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, MBM2k and MBM3400 with longer PEG spacer arm length showed better magnetic responsiveness than MBM, MBM400, and MBM1k. 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 MBM, MBM400, and MBM1k 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 MBM, MBM400, and MBM1k in the echogenicity measurements may be too low to accumulate at the target site. In contrast, when the magnet was applied, MBM2k and MBM3400 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 3: (A1–A5) The bright-field images of biotinylated MBs with different PEG chain lengths (no PEG spacer arm, MBb; with PEG400, MBb400; with PEG1000, MBb1k; with PEG2000, MBb2k and with PEG3400, MBb3400) and (B1–B5) the corresponding magnetic MBs (MBM, MBM400, MBM1k, MBM2k and MBM3400). (C1–C5) The fluorescent images of FITC-labeled magnetic MBs with different PEG chain lengths. The scale bar is 5 μm.
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 (\( B = 1.2 \) T) under sheer forces from 5 to 45 dyn/cm\(^2\). The biotinylated MBs were used as a control. Data represent mean ± 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. MBb1k and MBM1k (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 MBM2k and MBM3400 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 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 Fe3O4 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. It is hard to couple with the streptavidin-coated Fe3O4 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 MBBPM

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 (MBc). Its initial concentration was about 1.1 x 10^9 MBs/mL. BP nanosheets solution was added to the MBc 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 x 10^8 MBs/mL. The schematic of the finally obtained MBBPM was shown in 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 x 10^6/mL, pH 7.4, B = 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.
Figure 7A. As shown in the photographs in Figure 7B, the white MBc become black after adding BP nanosheets (i.e. MBBP) and subsequently magnetic NPs (i.e. MBBPM). The obtained MBBPM 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, MBBP and MBBPM 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 $-18.4 \text{ mV}$ and the MBc has a surface charge of 20.5 mV. After mixing them, the zeta potential of MBBP is negative. It further confirms the attachment of the BP nanosheets on the MBc. The obtained MBBPM possessed a surface charge of about $-12.6 \text{ mV}$. The particle size distribution of bare MBc is $2.7 \pm 0.2 \mu m$. The respective particle size distributions of MBBP and MBBPM are $3.6 \pm 0.1$ and $3.7 \pm 0.3 \mu m$, with the average particle sizes increased (Figure 7E).

To study the stability of the BP nanosheets loaded magnetic MBs, the remaining percentages of MBBP and MBBPM have been analyzed from as prepared to 1 h after the storage...
in the ambient condition. It can be observed in Figure 7F, MBBP is stable without obvious reduction. Moreover, through measuring the absorption of the subnatant in the MBBP solution, it shows no detected BP nanosheets desorption from MBBP (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/cm² 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
like the case of \( \text{MBPPM} \) 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 \( \text{MBPPM} \)**

To study the targeted photothermal therapy effect of the \( \text{MBPPM} \) in vitro, \( \text{MBPPM} \) and \( \text{MBP} \) 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 \( \text{MBP} \) (optical image of the MCF-7 breast cancer cells incubated with \( \text{MBP} \)) but numerous \( \text{MBPPM} \) (optical image of the MCF-7 breast cancer cells incubated with \( \text{MBPPM} \) 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 \( \text{MBPPM} \) can be seen before the NIR irradiation (optical image of the MCF-7 breast cancer cells incubated with \( \text{MBPPM} \) before NIR irradiation), but they diminish after the NIR irradiation (optical image of the MCF-7 breast cancer cells incubated with \( \text{MBPPM} \) after NIR irradiation), which are dynamited. After the treatments with different concentrations of \( \text{MBPPM} \) (from \( 1 \times 10^5 \) to \( 1 \times 10^7 \) \( \text{MBPPM/mL} \), \( 5 \times 10^{-5} \mu \text{g BP nanosheets/MB} \)), 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 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<sub>BBPM</sub> concentration increased to 1 × 10<sup>7</sup> MB<sub>BBPM</sub>/mL. The TEM analysis for the cellular uptakes of the MB<sub>BBPM</sub> (1 × 10<sup>7</sup> MB<sub>BBPM</sub>/mL) in MCF-7 cells was also performed, in order to assess the interactions of the MB<sub>BBPM</sub> 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<sub>BBPM</sub> 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 × 10<sup>7</sup> MB<sub>BBPM</sub>/mL was used in the further study of the in vitro photothermal therapy effect comparison between the MB<sub>BBPM</sub> and the BP nanosheets (Figure 10). It was found that, BP nanosheets and MB<sub>BBPM</sub> 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 W/cm<sup>2</sup> for 10 min did not lead to obvious killing effect. Extending the

![Figure 10](image-url)
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 and MB BPM have been obtained (Figure 11A). It can be seen that, after the injection of both MB 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 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. Since the US signal before the burst of the MBs comes from both the adherent MBs attracted by the magnetic field and the circulating 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 and MB BPM and it was much bigger of MB BPM (12.1 ± 1.2 a.u.) than that of MB (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/cm², 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 and MB BPM. (B) The echo–power–time profile of the tumor regions (ROI) 15 s before and after the burst of MB (blue dots) and MB BPM (grey dots) and their average values (MB: orange dots and MB BPM: purple dots). (C) The differential targeted enhancement (dTE) parameter for the MB and MB BPM. Data represent mean with SD, n = 5. *p < 0.05, **p < 0.01, ***p < 0.001.
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_Mₙ, MB_M₅₀₀, and MB_M₁₀₀₀ (lower than 50%) and poor magnetic responsiveness. In contrast, for the magnetic MBs with longer connecting PEG chain lengths—MB_M₂₀₀₀ and MB_M₃₄₀₀—the percentages were near 100%. Moreover, MB_M₂₀₀₀ 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-PEGx-biotin) with x = 0, 600, 1000, 2000, and 3400, were obtained from Smart-Elements (Austria) and anhydrous N-methyl-2-pyrrolidone (NMP) (99.5%) was bought from Ruixi Biological Technology Co. Ltd (Xi’an, China). The streptavidin-coated superparamagnetic Fe3O4 NPs (5 mg/mL, ~130 nm) were purchased from Zongkeleiming Technology Co. Ltd (Beijing, China). The bulk BP was obtained from 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-PEGx-biotin (x = 0, 600, 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 evacuated and octafluoropropane (C3F8) was introduced. Finally, a magnet (25 × 5 mm, ~1.2 T) was used to attract the magnetic MBs while the nonmagnetic MBs were redispersed in PBS for further characterization. The obtained magnetic MBs were denoted MB0, MB500, MB1k, MB2k, and MB3k, 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 strept-PEI600 was added in the MBs formulation to synthesize MB, with the molar ratio of DSPC, DSPE-PEG2000, DSPE-PEG2000-biotin, and strept-PEI600 to be 46:9:9:36. Then the BP nanosheets dispersion was mixed with the MBs. 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 unasorbed 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 an accugizer (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 obtained 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 BPM, 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 homemade parallel plate flow chamber assay. A total of 1 mL of magnetic MBs (with the same initial concentration of $1 \times 10^7$ MBs/mL) was injected into a vacuum parallel plate flow chamber (Glycotech 31-001, USA) by a stepping motor (Yuhui, China) through capillary tubing ($\rho = 0.15$ cm) at sheer forces of $5–45$ dyn/cm$^2$. A magnet ($25 \times 10 \times 5$ mm, $\sim 1.2$ T) was placed on the chamber throughout the experiments to capture the magnetic MBs, and then they were rinsed with 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 sheer 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 ($5 \times 10^6$ MBs/mL, 400 μL) 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/cm$^2$ 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 μL of DMEM (HyClone) supplemented with 10% (volume ratio) of fetal bovine serum and kept in an incubator consisting of 5% CO$_2$ at 37°C. Then they were incubated with MB$_{BP}$ 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/cm$^2$ 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 stained 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$_{BP}$, 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 Huafulang Biotechnology Co., Ltd. (Beijing, China). To establish the subcutaneous tumor model, MCF-7 cells ($5 \times 10^7$ MBs/mL, 150 μL) 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 MBs, and the MB$_{BP}$ ($n = 5$ for each group, $1 \times 10^7$ MBs/mL, 150 μL) 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 further allowed freely circulating MBs to replenish in tumors. The differential targeted enhancement (dTE) parameter was analyzed with the built-in Vevo Q software to evaluate the targeting effect of the MB$_{BP}$.

4.12 In vivo photothermal therapy study

To study the PTT efficacy of the MB$_{BP}$ in vivo, the tumor-mice were administrated intravenously the PBS, MB$_{BP}$, and the MB$_{BP}$ ($n = 5$ for each group, 150 μL) 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/cm$^2$, 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 $\times$ width$^2$/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$_{BP}$, the organs of heart, liver, spleen, lung, and kidney of the MB$_{BP}$ 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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