Targeted Soft Biodegradable Glycine/PEG/RGD-Modified Poly(methacrylic acid) Nanobubbles as Intelligent Theranostic Vehicles for Drug Delivery

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ABSTRACT: The development of multifunctional ultrasound contrast agents has inspired considerable interest in the application of biomedical imaging and anticancer therapeutics. However, combining multiple components that can preferentially accumulate in tumors in a nanometer scale poses one of the major challenges in targeting drug delivery for theranostic application. Herein, reflux-precipitation polymerization, and N-(3-(dimethylamino)propyl)-N′-ethylcarbodiimide-mediated amidation reaction were introduced to effectively generate a new type of soft glycine/poly(ethylene glycol) (PEG)/RGD-modified poly(methacrylic acid) nanobubbles with a uniform morphology and desired particle size (less than 100 nm). Because of the enhanced biocompatibility resulting from the glycine modification, over 80% of the cells survived, even though the dosage of glycine-modified polymeric nanobubbles was up to 5 mg/mL. By loading doxorubicin as an anticancer drug and perfluorohexane as an ultrasound probe, the resulting glycine/PEG/RGD-modified nanobubbles showed remarkable cancer therapeutic efficacy and a high quality of ultrasonic imaging; thus, the ultrasonic signal exhibited a 1.47-fold enhancement at the tumor site after intravenous injection. By integrating diagnostic and therapeutic functions into a single nanobubble, the new type of theranostic nanobubbles offers a promising strategy to monitor the therapeutic effects, giving important insights into the ultrasound-traced and enhanced targeting drug delivery in biomedical applications.

KEYWORDS: soft nanobubbles, glycine modification, targeting drug delivery, ultrasound imaging, theranostic platform

INTRODUCTION

Developing an effective biomedical imaging-guided cancer therapy has attracted much attention owing to its capabilities of both accurate evaluation of tumor conditions and therapeutic efficacy. Among various imaging modalities in biomedical diagnostics, ultrasound imaging has been extensively used, which is largely attributable to its intrinsic features such as safety, noninvasion, substantial tissue penetration, and low cost for visualization of internal tissues in real time. To enhance the ultrasound signals, ultrasonic contrast agents (UCAs), representing as aqueous dispersions of gas microbubbles sustained by a shell made of lipids, proteins, surfactants, or polymers, have addressed a broad spectrum of biomedical applications. Therefore, hollow polymer particles offer unprecedented advantages due to not only the structural properties (i.e., well-defined morphology, robust stability, and potential modification) but also the capacity to load multiple functional components (i.e., anticancer drugs and reporter probes) into the hollow chamber or shell for the combination of molecular imaging and therapeutics.

Along with enormous progress in the field of cancer therapy, polymer particles as the candidate of targeting drug carriers have successfully made their way from bench-top to preclinical studies. In this regard, polymer particles with sizes over 5.5 nm can avoid renal clearance, whereas particle sizes ranging from 10 to 200 nm allow to achieve the feasibility of passive accumulation in tumors due to enhanced permeability and retention (EPR) effect, implying that the polymer particles with the desired particle sizes enable to attain passive targeting reflected by higher efficiency of cell uptake in tumor tissue. To take advantage of the EPR effect, it is necessary to prolong the circulation time of drug carriers to enhance the possibility to reach tumor sites, instead of being captured by reticuloendothelial system. A promising solution is the covalent attachment of poly(ethylene glycol) (PEG), which has been well demonstrated by a variety of polymeric
nanohydrogels, liposomes, and polypeptides. On the other hand, active targeting strategies that conjugated with the targeting ligands on the surface of the drug carriers offer another important approach to pave the way for the modulation of therapeutic outcomes in tumor sites. Nevertheless, most of the existing UCAs should be fabricated in the micrometer scales to meet the requirement of the strong backscattering effect. Considering the mechanism of UCAs, the imaging signal generated from the vibration of microbubbles contains a gas core, which is strongly dependent on the diameters of the bubbles. When the bubble size matches the wavelength of the ultrasound, strong signals can be generated, leading to the rational engineering of the bubbles ranging between 2 and 8 μm to match the backscattering effect described above. However, conventional micron-sized bubbles are too large to get involved in EPR effect, which is unable to efficiently interstitial transport at tumor site, cells uptake, and further targeting to the cellular suborganelles. Given the limitation of the particle sizes to enhance tumor penetration via the EPR effect, especially preferable within 12–50 nm, it remains one of the major challenges in fabricating well-defined nanobubbles to achieve a larger population of nanobubbles gathering in tumor sites for an efficient therapeutic efficacy. Although many efforts have been devoted to explore the preparation of the nanoparticles for drug delivery and biomedical imaging, such as gold, silica (SiO2), manganese dioxide (MnO2), gadolinium (Gd), and some polymer-based nanoparticles, these nanoparticles have no cavity inside to match the wavelength of the ultrasound for ultrasound imaging. Recent advances by Liu’s group reported novel hollow Bi2Se3 nanoparticles with the diameter of around 35 nm, although it still seems difficult to obtain polymer-based nanobubbles with a such small size.

Herein, we prepared a new type of glycine/PEG/RGD-modified poly(methacrylic acid) nanobubbles with well-defined structural properties. The cytotoxicity of the as-prepared nanobubbles was greatly decreased by surface modification of glycine and PEG. Notably, the hydrodynamic diameter of the soft bubbles was diminished to less than 100 nm, which is beneficial for improving the efficiency of drug delivery and half-life circulation time compared with that of the reported microbubbles. Meanwhile, the size distribution of nanobubbles was still kept narrow to potentially enhance the sensitivity of contrast imaging. The size reduction of the microbubbles showed no impact on the intensity of ultrasound signal because the cavities of the bubbles were filled with perfluorohexane (PFH), enabling them to gasify and generate stronger signals at certain site compared with the polymeric nanobubbles without PFH. The biological evaluation of the imaging—therapy-integrated nanobubbles showed the desired theranostic effect both in vitro and in vivo. On the basis of the feasibility of multifunctional modification and codelivery of anticancer drugs and reporter probes, the new type of nanobubbles hold a great potential as a novel theranostic platform for cancer therapy in clinical application.

**MATERIALS AND METHODS**

**Materials.** Methacrylic acid (MAA), N-isopropyl acrylamide (NIPAm), 2,2-azobis(isobutyronitrile) (AIBN), N,N′-methylenebis(acrylamide) (MBAA), N-(3-dimethylaminopropyl)-N′-ethylcarboxy- mide hydrochloride (EDC-HCl), N-hydroxy succinimide (NHS), glycine, and doxorubicin (DOX), which is in the form of a hydrochloride salt, were all purchased from Shanghai Aladdin Chemistry Co. Ltd., the AIBN was recrystallized before use. N,N′-Bis(acryloyl)cystamine (BACy) was purchased from Alfa-aesar. PEG-NH2 (Mn = 1000) was provided by Huntsman Corporation. Vitamin E-TPGS1000 and phosphate buffer solution (PBS, pH 7.4) was provided by Sigma-Aldrich Chemical Co., Ltd. Cyanine 7-NHS was supplied by Life Technologies (Grand Island, NY). Perfluorooctylamine (PFH) was purchased from Energy Chemical Co. Ltd. Acetonitrile (AN) and ethanol were purchased from Shanghai Lingfeng Chemical Reagent Company. Dulbecco’s modified Eagle’s medium (DMEM), penicillin/streptomycin (P/S), trypsin, Hoechst 33342 nucleic acid stain, and fetal bovine serum were procured from Thermo Scientific (Waltham). The cell-counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Tokyo, Japan). Deionized water was used in all of the experiments.

**Preparation of PMAA Nanobubbles.** The nanobubbles were synthesized by reflux-precipitation polymerization. Typically, MAA, NIPAm, and AIBN (2 wt % to MAA) were dissolved with acetonitrile in a single-necked flask and boiled for 2 h under magnetic stirring to get the un-cross-linked PMAA nanoparticles (uNPs). The uNPs were purified by repeating ultracentrifugation (12 000 rpm for 15 min)/decantation/resuspension in acetonitrile for three times. Then, MAA, BACy, AIBN, and the uNPs were added to AN in a weight ratio of 3:0.9:0.06:1 with 2 mg/mL uNPs content. After the same operation as above for polymerization and purification, the PMAA nanoparticles with the core–shell structure were prepared. The resultant nanoparticles were dispersed in ethanol to dissolve the uNPs core, and the nanobubbles were purified with ultracentrifugation for following use.

**Surface Modification of the PMAA Nanobubbles.** The functional molecules, such as RGD, PEG, and glycine, were conjugated to PMAA nanobubbles by using the carbodiimide coupling method. Typically, PMAA nanobubbles (30 mg) were dispersed in acetonitrile (20 mL). After the addition of EDC-HCl (48 mg, 0.25 mmol), the mixture was heated to 70 °C and stirred for another 1 h. PEG-NH2 (60 mg, 0.06 mmol) was then added and the reaction mixture was stirred at 70 °C overnight. The obtained PEG-PMAA nanobubbles were purified by repeating ultracentrifugation (12 000 rpm for 3 min) and resuspension in PBS with ultrasonic for three times, excess glycine was added in the PEG-PMAA nanobubbles dispersion and stirred at room temperature overnight. After repeating ultracentrifugation (12 000 rpm for 15 min)/decantation/resuspension in deionized (DI) water with ultrasonic for three times, one part of the purified nanobubbles was mixed with c(RGDfK) conjugated TPGS1000 prepared before and the other part was mixed with TPGS1000 as a control, then the samples were freeze-dried for further use.

**Study of the Degradation of PMAA Nanobubbles.** The degradation of the PMAA nanobubbles can be measured by monitoring the turbidity of dispersion and molecular weight of PMAA. Briefly, 10 mg of PMAA nanobubbles was dispersed in 10 mL phosphate buffer (10 mM, pH 7.4) solution and then 10 mM GSH was added. The dispersion was placed in a shaking bed at 37 °C with a rotation speed of 200 rpm. At certain intervals, the scattering light intensity of the dispersion was determined by using dynamic light scattering (DLS). The turbidity was obtained by calculating the ratio of the scattering intensity of the degraded samples at 90° relative to that of the initial nondegraded sample (no GSH added). The molecular weight of the degraded polymers from the PMAA nanohydrogels in the presence of GSH was measured in 0.1 M NaNO3 aqueous solution by gel permeation chromatography (GPC). The degraded polymer solution was filtered through a 0.45 mm filter prior to its injection into the GPC.

**DOX Loading.** DOX was dissolved in a phosphate buffer solution (pH 7.4) of a certain concentration, and the dry nanobubbles were dispersed under stirring for 48 h at room temperature. More details of the postprocess were reported as earlier publication. The DOX-loaded nanobubbles were dried for further use.

**Cytotoxicity assay in Vitro Cell Culture.** The cytotoxicity was performed by cell-counting kit-8 (CCK-8) assay. The SKOV-3 cells and HEK293T cells were grown in McCoy’s 5A medium and Dulbecco’s modified Eagle’s medium (DMEM), respectively, supplementing with 10% heat-inactivated fetal bovine serum, 1%...
penicillin/streptomycin at 37 °C and 5% CO₂. The medium were changed every three days, and the cells were passaged by trypsinization before confluence. Cells were seeded onto 96-well plates (5,000 SKOV-3 cells or 10,000 HEK293T cells per well) and incubated for 24 h in a humified atmosphere of 5% CO₂ at 37 °C. The cells were washed with PBS, and the medium was replaced with a fresh medium containing the pure nanohybubbles, free drugs, or the drug-loaded nanohybubbles of known concentrations. After a certain time, the cells were washed with PBS and incubated in 100 μL medium containing 10 μL CCK-8 solution per well for another 1 h. The absorbance of each well was measured by a microplate reader (model FL 311, Bio-Tek Instruments Winooski, VT) at the wavelength of 450 nm. The results were expressed as the mean values of three measurements. Because the absorbance is proportional to the number of viable cells in the medium, the cell viability was determined by using a previously prepared calibration curve (Dojindo Co.).

To estimate the cellular uptake of nanobubbles, confocal laser scanning microscope (CLSM, Olympus, FV10-ASW) and flow cytometer (FCM, BD Biosciences) measurements were applied. Typically, the cells were seeded in 2 mL medium on glass bottom dish and grown overnight and incubated with free DOX and drug-loaded nanobubbles at a certain atmosphere as described before. Afterward, the cells were rinsed with PBS for three times, the nuclei were labeled by Hoechst 33342 solution (0.1 mg/mL in PBS, 1 mL per dish) for 10 min and rinsed with PBS three times. The samples were then visualized using CLSM. For FCM, the cells were seeded in 6-well plates at a density of 500,000 cells per well, the incubating conditions were same as those in the CLSM experiment. After washing three times with PBS, the cells were removed with trypsin and washed with PBS to remove the background fluorescence in the medium; finally, the cells were suspended in PBS for measurements.

In Vivo Fluorescence Tracking Experiments. The tagged molecule Cy7 (2.4 mg, 0.35 mM) was reacted with nanobubbles (30 mg) in DI water with EDC (48 mg, 25 mM) and NHS (28.8 mg, 25 mM) at room temperature for 24 h. The product was separated and purified by repeating ultracentrifugation and resuspension in DI water as mentioned before. In vivo real-time fluorescence imaging analysis was used to evaluate the in vivo distribution of the as-prepared nanobubbles. The BALB/c nude mice from SLAC were anesthetized by isoflurane. The dispersed suspension of the Cy7-labeled nanobubbles was injected through the caudal vein at a dose of 200 μL (0.1 mg/mL of Cy7). The mice were placed on an animal plate heated to 37 °C. The fluorescent images were captured at various time points using the Optical and X-ray small animal imaging system (Bruker, excitation: 720 nm, emission: 790 nm).

In Vivo Therapeutic Experiments. To evaluate the antitumor activity of DOX-loaded nanobubbles and free DOX, H22 tumor-bearing mice were randomly divided into four groups (six mice each group) and treated with DOX-loaded Gly/PEG/RGD-PMAA nanobubbles, DOX-loaded Gly/PEG-PMAA nanobubbles, free DOX, and physiological saline via tail vein injection, respectively, on days 0, 3, and 6, all of the samples is 5 mg/kg on DOX basis. The tumor size was measured by Vernier calipers, and the tumor volume (mm³) was calculated using V = a × b²/2, where a and b are the longest and shortest diameter axes of the tumors. The body weight of the mice was measured simultaneously as an indicator of systemic toxicity. All of the animal procedures were in agreement with the guidelines of the regional ethic committee for animal experiments in this work.

In Vivo Ultrasound Imaging. PFH was loaded into the DOX-loaded Gly/PEG/RGD-PMAA nanobubbles as reported before. Ultrasound images of the pure nanobubbles and the PFH-loaded nanobubbles under B mode were taken on a multimode photoacoustic/ultrasonic imaging system (Vevo LAZR, Fujifilm VisualSonics Inc.). Typically, the samples (2 mg/mL) were filled into the centrifuge tube (0.5 mL) and the transducer was coated with a ultrasound gel, all of the images were recorded as digital files for subsequent playback and analysis.

In Vivo Ultrasound Imaging. The H22 tumor-bearing mice were anesthetized by isoflurane and fixed at the multimode photoacoustic/ultrasonic imaging system. Then, 0.3 mL PBS of the DOX-loaded Gly/PEG/RGD-modified nanobubbles (2 mg/mL) was injected via tail vein. The tumor region of the mice was detected before and after the administration of the as-prepared nanobubbles.

Characterization. The morphologies of PMAA nanobubbles were measured on a transmission electron microscope (TEM, JEOL 1230) at an accelerating voltage of 75 kV. Hydrodynamic diameter and ζ potential were measured on a laser Doppler velocimeter (Malvern NanoZS). The zeta potentials were calculated using the Helmholtz-Smoluchowski equation: ζ = bV/2πa, where b is the Debye length, V is the potential difference, a is the hydrodynamic diameter, and ζ is the zeta potential.
potential of the nanobubbles were measured by dynamic light scattering (DLS) on an autosizer 4700 (Malvern) at a scattering angle of 90° under room temperature. The UV−vis spectra were obtained on a PerkineElmer Lambda 750 spectrophotometer.

■ RESULTS AND DISCUSSION

Fabrication and Characterization of the Polymeric Nanobubbles. The fabrication process of the nanobubbles (Nbs) is illustrated in Scheme 1. First, we employed a reflux-precipitation polymerization to prepare the un-cross-linked cores of PMAA or P(MAA-co-NIPAm), followed by surface coating of a new layer of the cross-linked PMAA to form a core−shell structure. Then, the cross-linked PMAA nanobubbles were obtained by dispersing the as-prepared core−shell particles in ethanol to selectively etch the un-cross-linked polymeric cores. Interestingly, the addition of some amount of NIPAm was identified to greatly reduce the core size of the particles in our synthetic system, which played a key role in shaping the small-sized nanobubbles.

Due to the key factors of shape and size of the drug carrier in application of cancer diagnosis and therapy, herein we pay more attention to the study of the related parameters. TEM images showed that the resulting PMAA nanobubbles displayed a series of definitive homogeneous bubble morphology via our flexible and convenient synthetic approach (Figure 1). The cavity of the nanobubbles possessed a delicate storage space to contain gas and other liquid ultrasonic agents for biomedical imaging.

The size of nanobubbles could be tailored by modulating the diameter of the un-cross-linked polymeric cores with the corresponding monomer ratio and concentration (Table S1). The PMAA nanobubbles ranged from ∼50 to ∼750 nm, as observed by TEM and FESEM (Figure S1, Table S2), in agreement with the results from dynamic light scattering (DLS) measurement, indicating that the tailored PMAA nanobubbles could be well dispersed in water with narrow size distribution. It is worth noting that the nanoparticles with sizes between 10 and 200 nm can take the advantage of EPR effect for an optimal cell-uptake effect; therefore, the smallest sample Nbs-1 ($D_h = 88$ nm) was selected for further investigation.

Degradation of the PMAA Nanobubbles. The disulfide bond in PMAA nanobubbles can be broken by GSH and lead to the disassembly of the nanobubbles.41 In this part, the nanobubbles of Nbs-3 were used as a model and the solid PMAA nanoparticles (Nps) with similar particle size (223 nm) were used as a control for the evaluation. It was found that Nbs-
3 had a faster degradation rate on account of the thin shell thickness (Figure S2a). The GPC result (Figure S2b) indicated that Nbs-3 was degraded into short oligomer with a narrow molecular distribution ($M_n = 1675$, $M_n/M_w = 1.10$), similar to that of Nps ($M_n = 1706$, $M_n/M_w = 1.12$).

**Surface Modification of the Nanobubbles.** As illustrated in Scheme S1, the procedure of conjugating poly(ethylene glycol) (PEG) and glycine (Gly) onto the surface of the nanobubbles include (i) activation of carboxyls from the PMAA nanobubbles by EDC in acetonitrile and (ii) formation of the amide bond in the presence of the amino-terminal PEG. By applying acetonitrile as the solvent to avoid excess hydrolysis of EDC, the EDC-activated PEG-PMAA nanobubbles were then transferred into a buffer solution (pH = 7.4) with excess glycine to remove the residual EDC conjugates on the nanobubbles. The whole process was monitored by measuring the hydrodynamic diameter and $\zeta$ potential. After grafting PEG onto the surface, the average diameter of the as-prepared nanobubbles increased from 88 to 240 nm, and the $\zeta$ potential varied from $-74.1$ mV to around $20$ mV (Figure 2a) owing to the occupation of EDC after reacting with carboxyls. Because the EDC conjugates were then replaced by glycine, the $\zeta$ potential returned to be negatively charge followed by slightly higher-than-initial values due to the replacement by PEG (Figure 2a), indicating a successful surface modification. Meanwhile, the diameters decreased to 182 nm as eliminating the aggregation of nanobubbles arising from the EDC modification. In addition, the surface charge of the nanobubbles changed to around $-5$ mV after loading DOX resulting from the electrostatic interactions between amine-carrying structure of DOX and the residual carboxyls.

The amount of PEG and glycine grafting onto the nanobubbles was estimated by elemental analysis. As shown in Table 1, the major elemental contents of nitrogen, carbon, sulfur, and hydrogen after surface modification of nanobubbles were listed compared with those of the original PMAA nanobubbles. We employed the following equations to evaluate the activation of carboxyls from modified PMAA nanobubbles.

The equivalence relation could be represented as follows

$$C = C_O X_O + C_P X_P + C_G X_G$$

$$N = N_O X_O + N_P X_P + N_G X_G$$

$$S = S_O X_O + S_P X_P + S_G X_G$$

Consequently, the mathematics equality, there was another equation

$$X_O + X_P + X_G = 1$$

where $C$, $N$, and $S$ represent the weight percentage of each element in the modified PMAA nanobubbles and the subscript letters $O$, $P$, and $G$ represent the component of the PMAA nanobubbles, PEG, and glycine, respectively. The capital letter $X$ is the weight percentage of the individual component in the modified nanobubbles. The weight percentage of each element in modified nanobubbles was equal to the weight percentage sum of the certain element in all of the three components.

Table 1. Elemental Contents in PMAA and Gly/PEG-Modified PMAA Nanobubbles

<table>
<thead>
<tr>
<th>Element Content (wt %)</th>
<th>N</th>
<th>C</th>
<th>S</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nbs (PMAA)</td>
<td>4.015</td>
<td>45.965</td>
<td>10.670</td>
<td>6.817</td>
</tr>
<tr>
<td>Gly/PEG-Nbs</td>
<td>5.244</td>
<td>48.635</td>
<td>4.970</td>
<td>12.24</td>
</tr>
</tbody>
</table>

"The weight percentages of nitrogen, carbon, sulfur, and hydrogen are represented by their initials, respectively.

Cytotoxicity and In Vitro Cell Uptake. To demonstrate whether the PEG/RGD/Gly-modified PMAA nanobubbles (PR-Nbs) can be efficiently internalized by cancer cells after loading DOX, we incubated the DOX-loaded PR-Nbs with SKOV-3 cells to evaluate the cytotoxicity and assess the cellular distribution. In our experiment, we found that the drug loading content (DLC) showed dependence on the molar ratio of the DOX to nanobubbles (Table S3). The DLC of the DOX-loaded PR-Nbs can be adjusted between 20 and 25% with good stability in PBS, whereas overloaded nanobubbles would induce the precipitation due to the poor dispensability of DOX in PBS. In addition, the DOX-loaded PR-Nbs showed a redox and pH dual-responsive drug release behavior (Figure S3), it is beneficial for selective drug release in tumor site. For detecting the targeted effect of RGD, we prepared the PEG/Gly-modified PMAA nanobubbles (P-Nbs) without RGD modification as a control group. As shown in Figure 3, the cytotoxicity of free DOX, DOX-loaded P-Nbs, and DOX-loaded PR-Nbs exhibited an increasing inhibition against SKOV-3 cells with an increased concentration. The DOX-loaded PR-Nbs showed a higher cancer cell killing potency compared with P-Nbs, which was similar to pure DOX after 24 and 48 h incubation.

The cellular distribution was analyzed by confocal laser scanning microscopy (CLSM). The nuclei of SKOV-3 cells were stained with Hoechst 33342 (blue). As shown in Figure 4a, after 12 h incubation, the red fluorescence from DOX was observed mainly in the nucleus, suggesting that the PR-Nbs could cross the cell membrane to release DOX in the cells and then rapidly diffuse into the nucleus. In contrast, the P-Nbs and free DOX only expressed low levels of fluorescence in the nucleus. It was probably attributed to the highly efficient RGD-receptor-assisted endocytosis of PR-Nbs, leading to the quick accumulation of PR-Nbs in the cells compared with P-Nbs and free DOX. In addition, quantitative study of the cellular
internalization and intracellular release of DOX was done by fluorescence-activated flow cytometry, showing that the DOX fluorescence intensity increased when the incubation time was prolonged, and the fluorescence intensity in PR-Nbs group was obviously stronger than that in P-Nbs group (Figure 4b).

**In Vivo Tracking and Therapeutic Effect.** To evaluate the accumulation effect of nanobubbles in tumor sites, PR-Nbs and P-Nbs labeled with Cy7 (5 wt % in 200 μL PBS solution) were injected into nude mice injected H22 tumor cells through the tail vein. As shown in Figure 5a, the fluorescence signals of Cy7 were observed throughout the whole body immediately after injection. The Cy7 molecule gradually accumulated in kidney and then bladder before being excreted through the urine quickly. However, the fluorescence intensity of P-Nbs and PR-Nbs was maintained for much longer time owing to the grafted PEG chains, which was beneficial to prolong the circulation life, although the initial fluorescence displayed a weaker intensity than the Cy7 group. After injection, the nanobubbles became gathered in organs such as liver, kidneys, and tumor sites in 2 h. After 12 h, the fluorescence intensity decreased gradually in the blood, proving that the injected nanobubbles were gradually cleared in the blood and accumulated in special organs. Then, the mice were sacrificed at 24 h to evaluate the biodistribution of the nanobubbles. The fluorescence intensity of the nanobubbles in tumor was much higher than that of free Cy7 molecule, indicating the effective accumulation and localization of the nanobubbles in the tumor sites (Figure S4). Comparing PR-Nbs with P-Nbs, we noticed that the fluorescence intensity of PR-Nbs in tumor sites was stronger than that of P-Nbs, which was largely attributable to the additional active targeting effect by RGD that could enhance their penetration into tumors and cell-uptake efficiency as previously demonstrated in the in vitro study. In contrast, the P-Nbs only relied on the EPR-dependent passive targeting to penetrate into tumor sites; thus, the PR-Nbs revealed a high level of preferential accumulation in the tumor sites.

Owing to the good tumor targeting ability, PR-Nbs loaded with DOX were capable of causing tumor suppression with minimal unexpected side effects. The experiment was conducted through intravenous injection via tail vein into H22 tumor-bearing mice by dividing into the following four treatments: saline (control), DOX, DOX-loaded P-Nbs, and PR-Nbs. The therapeutic efficacy was demonstrated by measuring the change of tumor volumes as shown in Figure 5b. Compared with the control group, the DOX-loaded P-Nbs
and PR-Nbs both led to effective suppression of tumors. Especially, the DOX-loaded PR-Nbs showed better tumor suppression ability than P-Nbs owing to the effective antitumor drug release resulting from high level of accumulation at tumor

Figure 5. In vivo real-time study of tumor accumulation and therapeutic efficacy. (a) Fluorescence images of nude mice injected with free Cy7, Cy7-labeled PR-Nbs, and P-Nbs. (b) The resulting relative tumor volumes and (c) body weight of Balb/c mice bearing H22 tumor in four experimental groups of saline (control), DOX, DOX-loaded P-Nbs (5 mg/kg DOX equiv), and PR-Nbs (5 mg/kg DOX equiv) at interval treatment time. The arrows represent the day on which the intravenous tail vein injection was performed.

Figure 6. In vitro ultrasound images of DOX-loaded PR-Nbs (a) without and (b) with PFH loading and in vivo ultrasound images of a mouse tumor (c) before and (d) after injection of PFH-loaded nanobubbles under B-mode. The scale bar indicated the depth from the probe head.
sites with efficient endocytosis induced by RGD. After 10 days, the tumors were almost diminished that cannot be observed. In contrast, the free DOX group only showed a limited tumor growth delay on account of the quick metabolization with no retention of DOX molecule in tumor. These results revealed the DOX-loaded PR-Nbs displayed more effective inhibition on tumor growth than DOX-loaded P-Nbs and free DOX group. Furthermore, there were no significant changes in the weight loss of mice after individual treatment by DOX-loaded P-Nbs or PR-Nbin, as shown in Figure 6c, indicating the minimal side effects. On the contrary, the mice injecting free DOX became thin and lazy and showed evidence of recovery after a long period of 14 days.

**Ultrasonic Imaging Investigation.** The DOX-loaded PR-Nbs were filled with perfluoropentane (PFH) in the inner cavities by a mild-infusion procedure (Scheme 1). As shown in Figure 6a,b, the PFH-loaded nanobubbles generated significantly positive signals in comparison with the nanobubbles without PFH in vitro. For in vivo study, the ultrasonic signal in tumor region displayed quite low intensity with the fuzzy structure inside the cancerous tissue before injection (Figure 6c). After injection of the nanobubbles into the mice blood vessel, the PFH-loaded nanobubbles gradually gathered into the tumor sites. When the ultrasonic probe was placed onto the tumor, the generated ultrasound energy gave rise to local overheating and induced PFH into small bubbles to produce a strong imaging signal, the tumor site became brightened with a clear boundary (Figure 6d). The ultrasonic signal exhibited a 1.47 (89.1/60.7-) fold enhancement at tumor site after intravenous injection compared with non-PFH nanobubbles, indicating that the soft nanobubbles could generate a strong ultrasonic signal with the combination of the filled PFH. About 4 h later, almost no signal can be detected (Figure S5). These results suggested that the PFH-loaded nanobubbles could effectively target the tumor tissues and enhance the brightness of tumor images under B-modes, making them a new developmental pathway to be potentially utilized as an efficient ultrasound contrast agent to trace the drug delivery.

**CONCLUSIONS**

In summary, we prepared a new type of glycine/PEG/RGD-modified PMAA nanobubbles with soft biodegradable polymeric shell, high DOX loading capacity, enhancement of ultrasound imaging, and biocompatibility. It is the first report of direct preparation of the monodisperse polymer nanobubbles by reflux-precipitation polymerization with the particle size less than 100 nm, which can take the advantage of EPR effect for targeting drug delivery. The usage of glycine could remove the redundant ester produced by EDC/NSH catalyzed step and improve the dispersibility of nanobubbles in aqueous solution. The glycine modification changed the component constitution of nanobubbles and greatly improved the biocompatibility. The DOX-loaded PR-Nbs showed a much better tumor therapeutic ability than P-Nbs; the tumors almost disappeared after DOX-loaded PR-Nbs injection. Furthermore, the ultrasound signal generated by PFH-loaded nanobubbles displayed strong intensity than observed in vivo, largely attributed to the filled PFH in the cavity of the nanobubbles that resulted in gasifying and then generating enhanced ultrasound signals at tumor sites. Therefore, by integrating diagnostic and therapeutic functions, the glycine/PEG/RGD-modified nanobubbles offer a promising nanoscaled theranostic platform to provide important insights into ultrasound-traced and enhanced targeting drug delivery in biomedical applications.

**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.7b11392.

Additional supporting scheme and figures (Scheme S1, Figures S1–S5) and tables (Tables S1–S3) for the modification of PMAA with poly(ethylene glycol) (PEG) and glycine (Gly); FESEM image of PMAA nanobubbles (Nb-6); investigation on the redox-induced degradation of Nbs-3 and Nps by turbidity measurements and GPC curve of the nanobubbles and nanoparticles after degradation for 24 h by 10 mM GSH; redox and pH dual-responsive drug-release profiles of the DOX-loaded PR-Nbs; quantitative analysis of Cy7 distribution in tumors and major organs examined at 24 h; the recipes for preparation of the un-cross-linked cores by reflux-precipitation polymerization; the size of the PMAA nanobubbles in TEM images and hydrodynamic diameters (Dh and PDI) of the swollen nanobubbles in water; the drug-loading content and efficiency (DLC/DLE) under a series of reaction ratio between drug and Nbs (PDF)

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