Stimuli-responsive biodegradable poly(methacrylic acid) based nanocapsules for ultrasound traced and triggered drug delivery system

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ABSTRACT

Ultrasound contrast agents (UCAs) have been investigated for echogenic intravenous drug delivery system. Due to the traditional UCAs with overlarge size (micro-scale), their reluctant accumulation in target organs and the instability have presented severe obstacles to the accurate response to the ultrasound and severely limited their further clinical application. Furthermore, elimination of drug carriers from the biologic system after their carrying out the diagnostic or therapeutic functions is one important aspect to be considered. The drug carriers with large sizes, avoiding renal filtration, will lead to increasing toxicity. In this present paper, we design and develop a new type of triple-stimuli responsive (ultrasound/pH/GSH) biodegradable nanocapsules, in which fill up with perfluorohexane, and the DOX-loaded PMAA with disulfide crosslinking forms the wall. These soft nanocapsules with uniform size of 300 nm can easily enter the tumor tissues via EPR effects. The PMAA shell has high DOX-loading efficiency (93.5%), the PFH filled can effectively enhance US imaging signal through acoustic droplet vaporization (ADV), ensuring diagnostic and image-guided therapeutic applications. What is more, the disulfide-crosslinked PMAA shell is biodegradable and thus safe for normal organisms. These merits enabled us optimize the balance of diagnostic, therapeutic and biodegradable functionalities in a multifunctional theranostic nanoplatform.

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1. Introduction

Cancer can affect various organs and occur in nearly every tissue in the body, which is one of the most challenging medical issues for handling [1]. The modern personal healthcare and pharmaceutical industries have raised some new concepts and requirements for the therapy of cancer, that is, it can be found at the early stages before the cancer metastasizes through the lymph systems and then the abnormal cells can be efficiently killed by chemotherapeutic agents [2,3]. The diagnostic applications of ultrasound (US) imaging were enormously popular now because ultrasound is non-invasive without ionizing radiation, real time and generally less expensive than other imaging techniques [4–6]. However, ultrasound imaging does not have a very sharp contrast and the detected area is sometimes buried and shadowed by other tissues. This problem has been resolved gradually by enhancing the quality of the ultrasound contrast agents (UCA) [7–9].

Ultrasound contrast agents are usually in the form of gas-filled microbubbles, which are typically 1–8 μm in diameter [8]. The gas core (air [10], perfluorocarbons [11] or sulfur hexafluoride [12]) is generally surrounded by a protein [13], lipid [14], surfactant [15], silica [16,17] or polymer shell [18,19] (from 2 to 500 nm thick), which can improve the stability against gas loss, dissolution, micrrobe coherence and produce a more standard size distribution. Meanwhile, ultrasound energy can increase the permeability of cell membranes and help agents penetrate through various tissues [20]. Besides, ultrasound wave can “trigger” drug release and control drug action and deposition in the region of disease while reducing undesired side effects in the healthy tissues [21]. Thus, a wide variety of application for drug delivery using UCAs are under investigation in numerous areas [22–28].

Due to the traditional UCAs with overlarge size, their reluctant accumulation in target organs and instability have presented severe obstacles to the accurate response to the ultrasound and severely limited their further clinical application [29]. Nano-sized drug
delivery system in diameter less than 700 nm is able to penetrate blood vessel walls and enter cancer cells outside of the vessels [30,31]. Recently, Shi et al. reported a nanometer-sized enhancement agent for ultrasound imaging, which consists of mesoporous silica nanocapsules (MSNs) as the carrier and an encapsulated temperature-sensitive perfluorohexane (PFH, with a favorable phase transition temperature of 56 °C) compound as a bubble generator [32]. Even though the enhanced US imaging through ultrasound-triggered acoustic droplet vaporization (ADV) [33] is encouraging, the strong adsorption ability of MSNs hinders the complete release of the drug and the biodegradability of MSNs also remains a controversial problem [34].

Elimination of drug carriers from the biologic system after their carrying out the diagnostic or therapeutic functions is one important aspect to be considered [35,36]. It has been found that the hydrodynamic size required for renal clearance is very small (<3.5 nm) [37], the drug carriers with large sizes, avoiding renal filtration, will lead to increasing toxicity [38,39]. Therefore, in order to maximize drug-loading efficiency and reduce the toxicity of the carrier, developing nanoscale biodegradable multifunctional microbubbles as contrast agent for ultrasound directed drug delivery are highly desirable. Very recently, our group reported a new type of disulfide cross-linking biodegradable poly(methacrylic acid) (PMAA)-based nanohy-drogs for controlled drug release [40]. These nanohydrogels not only had excellent colloidal stability and high loading efficiency of doxorubicin (DOX), but also could be degraded into short polymer chains using water-soluble reducing agents.

Based on our previous work, we designed and developed a new type of uniform biodegradable nanocapsules, in which fill up with perfluorohexane, and the disulfide cross-linkage PMAA with DOX as wall (noted as PMAA-PFH nanocapsules). This design enabled us to achieve the optimal balance among diagnostic, therapeutic and biodegradable functionalities in a three-in-one theranostic nanoplatorm.

2. Materials and methods

2.1. Materials

Methacrylic acid (MAA) were purchased from Sigma Aldrich and distilled under reduced pressure prior to use. Perfluorohexane (PFH) and cystamine dihydrochloride were purchased from Acrors Organics. Acryloyl chloride, glutathione (GSH) and ethanol were purchased from Shanghai Aladdin Chemistry Co. Ltd. Doxorubicin (DOX), in the form of a hydrochloride salt, was obtained from Beijing Huafeng United Technology Company. 2, 2-azobisisobutyronitrile (AIBN) was obtained from Sinopharm Chemical Reagent Company and recrystallized from ethanol. Acetonitrile (AN) was purchased from Shanghai Lingfeng Chemical Reagent Company and dried from azeotropic distillation. Deionized water was used in all our experiments.

2.2. Synthesis of disulfide crosslinker of N, N-bis(acryloyl)cysteamine (BACy)

The disulfide crosslinker was synthesized according to the published paper [41]. In a typical reaction, cystamine dihydrochloride (5.630 g, 0.025 mol) was dissolved in water (25 mL) and added to a four-necked, 250-mL flask equipped with a stirrer, a thermometer, and two dripping funnels. After the mixture was cooled to 0 °C, an acryloyl chloride (4.526 g, 0.05 mol) solution in dichloro-methane (5 mL) and an aqueous NaOH solution (4.0 g, 0.1 mol; 10 mL water) were added simultaneously and dropwise slowly under stirring for more than 1 h while the temperature was kept at 0 °C. After the addition, the reaction mixture was stirred at room temperature for more than 6 h. The organic phase was separated, extracted with dichloromethane, and subsequently dried over anhydrous Na2SO4, after that, the solvent was removed under vacuum. The raw BACy product was purified by recrystallization from ethyl acetate/heptane (1/2:1 volume) mixture.

2.3. Preparation of biodegradable PMAA nanocapsules

First, the uncrosslinked PMAA microspheres (u-PMAA) were prepared by using the distillation-precipitation polymerization: MAA (0.5 g, 5.808 mmol), and AIBN (16.0 mg, 0.1 mmol) were dissolved in 40 mL of acetonitrile (AN) in a dried 50-mL single-necked flask with the aid of ultrasound for 10 min. Then, the flask submerged in a heating oil bath was attached with a fractionating column, Liebig condenser and a receiver. The reaction mixture was heated from ambient temperature to the boiling state at 10 min and the reaction was ended after about 20 mL of acetonitrile was distilled from the reaction mixture within 1 h. The obtained u-PMAA microspheres were separated and purified for three times by repeating ultra-centrifugation (12000 rpm for 10 min) and dispersion cycle in AN with ultrasonic baking. Then, the u-PMAA microspheres were dispersed in AN as seeds to synthesize disulfide-crosslinked PMAA (d-PMAA) coated u-PMAA/PMAA core–shell microspheres by using the same distillation-precipitation polymerization: u-PMAA microspheres (0.1 g), BACy (0.2 g, 0.77 mmol) MAA (0.5 g, 5.808 mmol), and AIBN (16.0 mg, 0.1 mmol) were dissolved in 40 mL of acetonitrile (AN) in a dried 50-mL single-necked flask. Finally, the PMAA nanocapsules were obtained by dispersed in ethanol for 3 h to remove uncrosslinked PMAA cores and then freeze-dried for 48 h in vacuum freeze-dryer.

2.4. Preparation of DOX-Loaded PMAA-PFH nanocapsules

DOX was chosen as a model drug to investigate the drug loading and controlled release behavior and PMAA nanocapsules with 40% crosslinking degree (PMAA-40) were chosen as drug carriers. Typically, 10 mg of dry PMAA-40 nanocapsules and 6 mg of DOX were dispersed in 20 mL of phosphate buffer solution (PBS) (pH 7.4) under stirring for 24 h at room temperature. The dispersion was then centrifuged to collect DOX-loaded PMAA-40 sediments and washed with PBS (pH 7.4) for three times to remove the surface adsorbed DOX. The sample was obtained by centrifugation and freeze-dried for 48 h in vacuum. The DOX mass loaded into PMAA-40 was calculated by subtracting the mass of DOX in the total supernatant from the mass of the drug in the initial solution measured by a UV visible spectrophotometer at 480 nm and a standard DOX calibration curve.

In the absence of water, 50 mg DOX-loaded PMAA-40 nanocapsules stored in a 5 mL bottle were infused dropwise with 150 mL highly echogenic PFH liquid. Thereafter, the bottle was filled with soft tape and capped tightly to prevent the volatilization of PFH. 2 min sonication in ice water was then performed to facilitate the PFH loading in nanocapsules. Then DOX-loaded PMAA-PFH was dispersed in 25 mL deionized water under slight magnetic stirring for 2 h at room temperature. After centrifuged at 10,000 rpm for 5 min, the obtained DOX-Loaded PMAA-PFH nanocapsules were dispersed in 10 mL deionized water and then freeze-dried to immobilize the enhancement agent system during the transportation and experimentation.

2.5. Redox-triggered disassembly of PMAA nanocapsules

The turbidity change of the PMAA-40 nanocapsules in response to reducing agents GSH was monitored by dynamic light scattering (DLS) measurement. Briefly, 5 mg PMAA-40 was poured into 10 mL phosphate buffer (10 mM, pH 7.4) solution and then 10 mM GSH was added. The solution was placed in a shaking bed at 37 °C with a rotation speed of 200 rpm. At predetermined intervals, samples were collected and their scattering light intensities were determined by using DLS. The turbidity was obtained by calculating the ratio of the scattering intensity at 90° of the irradiated samples relative to that of the initial non-degraded sample (no GSH added). The molecular weights of the degraded polymers from the PMAA nanocapsules in the presence of DTT or GSH was measured in 0.1 M NaNO3 aqueous solution by Gel Permeation Chromatography (GPC). The degraded polymer solution was filtered through 0.45 mm filter without strong pressing prior to its injection into the GPC.

2.6. In vitro DOX release

In vitro DOX release

The DOX-loaded PMAA-PFH (10 mg) were dispersed in 10 mL of 2 different buffer solutions (phosphate buffer/pH 7.4; acetate buffer/pH 5.0) and the dispersion was divided into five equal aliquots. Each 2 mL of the aliquot sample was then transferred into a dialysis bag (molecular weight cut off 14,000), which were dialyzed against 80 mL of the corresponding buffer (pH 7.4 or 5.0) with or without 10 mM GSH and ultrasound irradiation (with frequency of 4.0 MHz, pulse repetition frequency (PRF) of 1 kHz, pressure amplitude of 1.0 MPa and MI = 0.6 at 50% power) and gently shaken (200 rpm) at 37 °C, respectively. The drug release was assumed to start as soon as the dialysis bags were placed into the reservoir. At predetermined intervals, 3 mL of the solution was obtained periodically from the reservoir, and the amounts of released DOX were analyzed by UV–vis at 480 nm. For keeping a constant volume, 3 mL of fresh buffer medium was added back to the reservoir after each sampling.

2.7. Confocal laser scanning microscopy (CLSM) observation

The cellular uptake of the DOX-loaded PMAA-PFH was confirmed by confocal laser scanning microscopy (CLSM) observation. HeLa cells were seeded in 6-well culture plates (a clean cover slip was put in each well) and grown for 24 h. Then, the cells were incubated with DOX-loaded PMAA-PFH at 37 °C for 2 h and 18 h. Thereafter, the cells were rinsed with PBS three times, fixed with 2.5% formaldehyde (1 mL per well) at 37 °C for 10 min, and then rinsed with PBS three times again. To perform nucleus labeling, the nuclei were stained with DAPI solution (from Molecular Probes, 20 mg mL−1 in PBS, 1 mL per well) for 10 min and then rinsed with
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2.8. In vitro cell assays

The cytotoxicity assay was performed by cell counting kit-8 (CCK-8) assay [42]. The HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U mL$^{-1}$ penicillin and 100 U mL$^{-1}$ streptomycin at 37 °C, 5% CO$_2$. The media were changed every three days, and the cells were passaged by trypsinization before confluence. Cells were seeded onto 96-well plates (10,000 cells well$^{-1}$) and incubated for 24 h in a humidified atmosphere of 5% CO$_2$ at 37 °C. The HeLa cells were washed with PBS, and the medium was replaced with a fresh medium containing the pure PMAA-40 nanocapsules, free drugs (DOX), or the drug-loaded PMAA-PFH of known concentrations. After 24 h, the cells were washed with PBS and incubated in 100 μL DMEM containing 10 μL CCK-8 solution for another 1 h. The absorbance of each well was measured by a micro-plate reader (model FL311, Bio-Tek Instruments, Winooski, VT, USA) at the 450 nm wavelength. The results were expressed as mean values of four measurements. Then the cells were washed with PBS and incubated in fresh medium for an additional 24 h and measured again. Since 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 (Dejing Co.). The cytotoxicity analysis of pure PMAA-PFH droplets to HEK 293 cells was conducted in the same way mentioned above.

2.9. In vitro ultrasound imaging

Ultrasound images in vitro with PBS control, pure PMAA nanocapsules and PMAA-PFH under B mode and power Doppler mode were carried out on a color digital ultrasonic diagnostic apparatus LOGIQ Book XP (GE, America). Typically, phosphate buffered solution (PBS, 20 mL) with constant concentration of samples (2 mg mL$^{-1}$) was filled into a dialysis bag (2.5 cm × 10.0 cm). The ultrasound imaging was achieved by using 8L-RS probe (4–10 MHz, linear array) with frequencies of 4 MHz, pressure amplitude of 1.0 MPa, FRF of 100 Hz and MI of 0.4 for B mode and 1.1 for power Doppler mode at 40% power. The transducer was coated with ultrasound gel to avoid air back-ground. All images were recorded as digital files for subsequent playback and analysis. The ‘average gray scale’ was calculated by averaging the gray scale levels of all pixels within an indicated region of interest (ROI).

2.10. In vivo ultrasound imaging

Animal procedures were in agreement with the guidelines of the regional ethic committee for animal experiments. The healthy rat and the pancreatic tumor bearing nude mouse (around 200–300 g of body weight) were obtained from Shanghai laboratory animal research center and were fasted for 24 h before experiments. The mice were anesthetized using 10% chloral hydrate and fixed at the ultrasound therapy system. The scanning parameter settings were the same as for the in vitro experiments. Then, 1 mL PBS blank and PBS of PMAA-PFH (0.5 mg/mL) were injected via tail vein. The livers and tumor regions of the nude mice were scanned before and after the administration of the as-prepared PMAA-PFH nanocapsules.

2.11. Measurement

HRSEM images were performed on a TS-5136MM (TESCAN, Czech) scanning electron microscope at an accelerating voltage of 20 kV. Samples dispersed at an appropriate concentration were cast onto a glass sheet at room temperature and sputter-coated with gold. TEM images were obtained on an H-600 (Hitachi, Japan) transmission electron microscope at an accelerating voltage of 200 kV. Samples dispersed at an appropriate concentration were cast onto a carbon-coated copper grid. FTIR spectra were recorded on a Magna-550 (Nicolet, USA) spectrometer. The samples were dried and mixed with KBr to be compressed to a plate for measurement. TGA data were obtained with a Pyris-1 (Perkin–Elmer, USA) thermal analysis system under a flowing nitrogen atmosphere at a heating rate of 10 °C/min from 100 to 800 °C. Hydrodynamic diameter measurements were conducted by dynamic light scattering (DLS) with a ZEN3600 (Malvern, UK) Nano-ZS instrument.

3. Results and discussion

3.1. Design and synthesis of PMAA-PFH nanocapsules

The preparation of biodegradable drug-loaded PMAA-PFH nanocapsules and the concept of ultrasound traced and triggered drug delivery system are demonstrated in Scheme 1. Firstly, the uncrosslinked PMAA (u-PMAA) microspheres as the seeds were uniformly coated with a new layer of disulfite-crosslinked PMAA (d-PMAA) to form u-PMAA@d-PMAA core–shell structure by using distillation-precipitation polymerization. Secondly, the PMAA nanocapsules were prepared by dispersing u-PMAA@d-PMAA in ethanol to selectively dissolve u-PMAA. Thirdly, the anti-carcinogen DOX was loaded in shells of PMAA microbubbles through strong electrostatic interaction between the carboxyl groups of PMAA and the amino groups of DOX. Finally, the DOX-loaded PMAA nanocapsules were filled with the temperature sensitive perfluoropentane (PFH) in the inner cavities by a mild infusion procedure (Scheme 1A). When the PMAA-PFH nanocapsules were injected into animal vessel (Scheme 1B), the ultrasound energy could induce PFH into small bubbles to create strong imaging signal and increase the permeability of vessel to help drugs penetrate through vessel to tumor cells. While the nanocapsules entered cancer cells, the drug could be triggered and effectively released under ultrasound wave and glutathione (GSH) reduction condition. Meanwhile, the PMAA shells could be degraded into short polymer chains to be eliminated from the body.

3.2. Characterization of PMAA nanocapsules

The transmission electron microscope (TEM) imaging of u-PMAA@d-PMAA microspheres (Fig. 1B) showed a darker and more uniform morphology compared with u-PMAA microspheres (Fig. 1A) due to the robust crosslinked shells. The PMAA nanocapsules obtained by etching u-PMAA@d-PMAA microspheres exhibited excellent monodispersity with an average diameter of 300 nm and shell thickness of 25 nm (Fig. 1C, D). Although the nanocapsules were prone to collapse during the sample preparation under high vacuum (TEM conditions), they maintained good spherical morphology under scanning electron microscopy (SEM) conditions. The dynamic light scattering (DLS) measurement showed that the PMAA nanocapsules could be well swollen in water and owned hydrodynamic diameter of 810 nm with a narrow size distribution (Fig. S1).

We further investigated the effect of crosslinking degree on microbubbles morphology. As shown in Fig. 2, when the BACy crosslinker concentration was 10 wt%, the shell of nanocapsules was very thin and all the nanocapsules collapsed into transparent gossamer microbubbles. As the crosslinker concentration increased from 20 wt%, 30 wt% to 40 wt%, the shells of nanocapsules became thicker and the morphologies of nanocapsules also became integrated spheres from collapsed microbubbles. All nanocapsules had narrow size distribution in absence of any secondary-initiated particles.

The FTIR data displayed that the typical amide I (1650 cm$^{-1}$) and amide II (1550 cm$^{-1}$) bands of BACy crosslinker and the C=O stretching vibration of MAA were all found in the PMAA nanocapsules (Fig. 3A), suggesting the nanocapsules consisted of BACy crosslinker and MAA monomer, rather than only MAA monomer. As the crosslinker concentration increased from 10, 20 and 30 to 40 wt%, the intensity of amide bands of BACy became stronger and stronger (Fig. S3B). Thermal gravimetric analysis (TGA) data further suggested the different amounts of the crosslinker in PMAA nanocapsules with different crosslinking degrees. The PMAA nanocapsules revealed lower pyrolysis temperature compared with uncrosslinked PMAA and u-PMAA@d-PMAA microspheres (Fig. 4A). Meanwhile, pyrolysis temperature of PMAA nanocapsules became lower and lower with the crosslinking degree increasing (Fig. 4B). This may be that the cross-linkages containing disulfide bonds were more inclined to pyrolyze.
Scheme 1. (A) Schematic representation of the preparation of DOX-loaded PMAA-PFH nanocapsules. (B) Schematic procedure for imaging-guided ultrasound triggered drug delivery to tumors using biodegradable PMAA-PFH nanocapsules.

Fig. 1. TEM images of (A) uncrosslinked PMAA microspheres (u-PMAA) and (B) u-PMAA@d-PMAA core–shell microspheres. HR-TEM images (C) and HR-SEM (D) images of PMAA-40 (crosslinking degree 40%) nanocapsules. All scale bars are 500 nm.
nanocapsules with 40% crosslinking degree (noted as PMAA-40) as drug carrier and ultrasound contrast agent. The degradation behavior of PMAA-40 nanocapsules was monitored by the turbidity measurement. Upon the addition of water-soluble GSH as a reducing agent, the skeleton network of the nanocapsules was quickly degraded into individual polymeric chains, and the appearance of the milky emulsion changed gradually to a clear solution within 50 min, while the structure of the nanocapsules was very stable in the solution without GSH addition. The relative turbidity (the real-time turbidity compared with the original data) decreased quickly in the first hour, and then changed slowly (Fig. 5A). Meanwhile, the relative turbidity was almost unchanged in the absence of GSH. The molecular weight of degraded PMAA-40 nanocapsules was measured by GPC (Fig. 5B). The GPC result indicted the in nanocapsules were degraded into very small polymer chains with low molecular weight ($M_n = 1150$) and narrow molecular weight distribution ($M_w/M_n = 1.06$), which was in accordance with our previous report [40]. These degradation experiments suggested that the PMAA nanocapsules could be biodegradable in vivo so that they could reduce toxicity produced by their accumulation in the body. These biodegradable PMAA nanocapsules may be a great potential platform for ultrasound imaging and drug delivery.

3.4. Loading of DOX and in vitro release kinetics

Owing to that the carboxyl group has strong electrostatic interactions with amino group, we chose DOX as model anticancer drug to evaluate the capability of PMAA-40 nanocapsules as drug carriers. In our experiment, both high drug loading capacity (36 wt %) and excellent drug loading efficiency (93.5%) could be easily obtained.
In order to enhance the signal of ultrasound imaging and achieve image-guided drug delivery, we filled DOX-loaded PMAA-40 nanocapsules with temperature-sensitive PFH (PMAA-PFH nanocapsules), which could generate highly echogenic phase-transitioned bubbles under ultrasound wave irritation. Accounting for the varying pH and redox potential between the extracellular and intracellular environments, as well as different external stimulation, the drug-release experiment was conducted by subjecting DOX-loaded PMAA-PFH nanocapsules to different buffer solutions with pH values of 7.4 and 5.0 and different GSH concentrations and ultrasound conditions. As shown in Fig. 6A, the amount of released DOX is only 11% at pH 7.4 without GSH or ultrasound over a period time of 24 h. When adding 10 mM GSH (mimicking the intracellular environment with GSH concentration about $2 \times 10^4$ mM), it showed a quick drug release, the DOX release reached over 70 wt% after 5 h and the cumulative release was approximately 82 wt% after 24 h. Further, under ultrasound irritation, this system showed incredibly rapid drug release rate and reached 93% after 15 min.

Under acidic pH conditions (pH 5.0), the DOX drug release was much faster than in the physiological solution (Fig. 6B), indicating the sensitivity of PMAA shell to endo-/lysosomal pH. In the absence of GSH, DOX released from the carriers in pH 5.0 was about 30 wt% after 24 h, which was double the DOX release in a physiological solution after the same period. In the presence of 10 mM GSH but without ultrasound, the DOX release increased to over 97 wt% within the initial 3 h. What is more, the amount of drug release reached to 98 wt% within 5 min during ultrasound triggered nanocapsules destruction. This ultrasound-triggered drug release could provide an efficient approach for drug delivery and cancer chemotherapy.

3.5. In vitro cell assays

To further demonstrate whether PMAA-PFH nanocapsules can be efficiently internalized by cancer cells and deliver drugs in cells, we incubated HeLa cells with DOX-loaded PMAA-PFH nanocapsules for 2 h and 18 h at 37 °C, and the cellular distribution was evaluated by confocal laser scanning microscopy (CLSM) analysis. As shown in Fig. 7A, after 2 h of incubation, the red fluorescence from DOX was observed in cytoplasm along with little red signal in the cell nucleus, suggesting the PMAA-PFH nanocapsules crossed the cell membrane and DOX molecules were released and localized within the cytoplasm. What is more, with the incubation time increasing to 18 h (Fig. 7B), the red fluorescence was observed in both the cytoplasm and the cell nucleus. The results suggested the efficient intracellular delivery and release of DOX by PMAA-PFH carriers.

Standard CCK-8 assay was adopted to assess the cytotoxicity of pure PMAA-40, DOX-loaded PMAA-PFH and free DOX at different concentrations. The experimental data in Fig. 8 showed that, after
incubation with the HeLa cells 24 h and 48 h in a wide range of pure PMAA-40 concentration (0.1–100 mg mL\(^{-1}\)), no cytotoxicity could be detected even at relatively high concentrations. In contrast, free DOX and DOX-loaded PMAA-PFH exhibited an increasing inhibition against HeLa cells with an increased concentration. The cell viability for the DOX-loaded PMAA-PFH was similar to pure DOX after 24 and 48 h incubation.

The in vitro cell experiments for normal HEK 293 cell line were also conducted for a preliminary assessment of the cytotoxicity of the pure PMAA-PFH droplets (Fig. S2). The viability of control cells was assumed to be 100%. After 24 h, there is no cytotoxicity against HEK 293T cells in diverse concentrations. When the incubation time was prolonged to 48 h, the cell viability still remained above 95% at a very high concentration of 200 mg mL\(^{-1}\). These results implied that the biodegradable PMAA carriers had the potential to be used as ultrasound targeted antitumor nanomedicine and enhance the drug activity.

### 3.6. In vitro US imaging evaluation

For investigating the contrast-intensified US imaging of DOX-loaded PMAA-PFH nanocapsules, the PBS control, pure PMAA-40 and DOX-loaded PMAA-PFH nanocapsules in PBS with the same concentration were wrapped up by dialysis bags for the following US imaging in vitro under 2D gray-scale ultrasound (B-mode) and power Doppler ultrasound imaging mode (Fig. 9). As we can see, the introduction of pure PMAA-40 and PMAA-PFH showed backscatter signal enhancement under both B-mode and power Doppler ultrasound imaging mode with respect to the control phosphate buffered solution (PBS), which is almost transparent to ultrasound. Furthermore, such contrast enhancement of PMAA-PFH was much greater than that of pure PMAA-40. To better characterize the quantity of imaging signal, we further measured the corresponding average gray values in both B-mode and power Doppler mode. Remarkable increase in average gray scales for PMAA-PFH, compared to pure PMAA-40 and the PBS control, were detected under B-mode (with the values of 89, 42 to 0) and power Doppler mode (with the values of 173, 62 to 4). Such a significant enhancement in US signal using PMAA-PFH was attributed to the high encapsulation capacity of PFH.

### 3.7. In vivo US imaging evaluation

We assessed the imaging ability of PMAA-PFH nanocapsules as the contrast agents for in vivo US imaging using healthy rat under B-mode and power Doppler mode. As shown in Fig. 10, the PMAA-PFH
nanocapsules have generated significantly positive contrast enhancement in the liver tissue. One minute after injection of the agents, we could observe enhanced ultrasound imaging, indicating the real-time property of ultrasonography.

To further evaluate the tumor imaging of these nanocapsules in vivo, the ultrasound imaging under different modes after intravenous injection of PMAA-PFH in pancreatic tumor-bearing nude mouse were evaluated as shown in Fig. 11. Before injection, the imaging signal of tumor region was very low and the boundary of cancerous tissue and liver was very fuzzy. After post-intravenous injection of PMAA-PFH nanocapsules, both the tumor and the surrounding liver tissue became brightened and the boundary became clear, indicating that the nanocapsules could enter the tumor tissues via enhanced permeability and retention (EPR) effects [43] due to the leaky vasculature and poor lymphatic drainage in tumor. These results suggested that the PMAA-PFH nanocapsules could effectively enhance the tumor brightness contrasting to the surrounding normal tissues under varied modes, and be potentially used as an efficient US contrast agent to trace drug delivery.

4. Conclusion

We have successfully developed a US traced and triggered drug delivery system using a new preparation method, the superiority of their US contrast efficiency and excellent drug-delivery capacity has been confirmed. In comparison with previous reported ultrasound targeted drug release systems, our system has four advantages as following: (1) the PMAA-PFH nanocapsules are very uniform, soft and small (300 nm), which can easily enter the tumor tissues via EPR effect; (2) the PMAA shell has high DOX-loading content (36 wt%) and great drug loading efficiency (93.5%) by strong electrostatic interactions between carboxyl groups and amino groups, and the loading drug can be quickly released (<5 min) under US conditions; (3) the PFH filled can effectively

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**Fig. 8.** Anti-tumor activity of DOX-loaded nanocapsules, PMAA-40 nanocapsules and free DOX as a function of DOX dosages for (A) 24 and (B) 48 h incubation of HeLa cells. The concentration of PMAA-40 nanocapsules (blank control) was shown on the top x-axis.

**Fig. 9.** In vitro US imaging for PBS control, PMAA-40 nanocapsules and PMAA-PFH nanocapsules under B-mode and power Doppler mode.
enhance US imaging signal through acoustic droplet vaporization (ADV), ensuring diagnostic and image-guided therapeutic applications; (4) what is more, the disulfide-crosslinked PMAA shell is biodegradable and thus safe for normal organisms. All these merits enabled us attain the final goal for optimizing the balance of diagnostic, therapeutic and biodegradable functionalities in a three-in-one theranostic nanoplatform.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.biomaterials.2013.11.057.

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