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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 content (36 wt%) and great drug 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.

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, microbubble coalescence 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

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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 carriers 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 (<5.5 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-linked biodegradable poly (methacrylic acid) (PMAA)-based nanohydrogels 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 nanoplatform.

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 Acros 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 with calcium hydride and purified by distillation before use. Dichloromethane, ethyl acetate and heptane were obtained from Shanghai Chemical Reagents Company and used without a further purification. Deionized water was used in all our experiments.

2.2. Synthesis of disulfide crosslinker of N, N-bis(acryloyl)cystamine (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 Na_2SO_4, after that, the solvent was removed under vacuum. The raw BACy product was purified by recrystallization from ethyl acetate/heptane (1/2 in 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 within 30 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 bathing. Then, the u-PMAA microspheres were dispersed in AN as seeds to synthesize disulfide-crosslinked PMAA (d-PMAA) coated u-PMAA@d-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 total 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 μL highly echogenic PFH liquid. Thereafter, the bottle was lidded with scotch 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 PBS to cap sensitive PFH and 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 weight of the degraded polymers from the PMAA nanocapsules in the presence of DTT or GSH was measured in 0.1 M $NaNO_3$ 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

Typically, 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 irritation (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. The HeLa cells were seeded in 6-well culture plates (a clean cover slip was put in each well) and grown, overnight, as a monolayer, and 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 was stained with DAPI solution (from Molecular Probes, 20 mg mL^{-1} in PBS, 1 mL per well) for 10 min and then rinsed with