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A novel bubble-forming material for preparing hydrophobic-agent-loaded bubbles with theranostic functionality

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ABSTRACT

In the present study, a new bubble-forming material (carboxymethyl hexanoyl chitosan, CHC), together with superparamagnetic iron oxide (SPIO) nanoparticles, was employed to prepare image-guided bubbles for efficiently encapsulating and delivering hydrophobic agents to kill tumor cells. The results showed that CHC could be used for preparing not only micronized bubbles (CHC/SPIO MBs) to exhibit ultrasound imaging functionality but also nanosized bubbles (CHC/SPIO NBs) to exhibit magnetic resonance T_2 image contrast. It was found that the amounts of SPIO nanoparticles and hexane during preparation process were the key factors to obtaining CHC/SPIO NBs. Most importantly, under in vitro cell culture conditions with the same amount of camptothecin (CPT) and therapeutic sonication, CPT-loaded CHC/SPIO NBs demonstrated more significant transcellular delivery and cytotoxicity than free CPT. Subsequently, an intratumoral injection was proposed for the in vivo administration of hydrophobic-agent-loaded CHC/SPIO NBs. After injection, the distribution of a hydrophobic dye (DiR, an agent with near-infrared (NIR) fluorescence used as a model drug) released from the CHC/SPIO NBs was tracked by an NIR imaging technique. A significant tumor-specific accumulation was observed in the mouse that received the DiR-loaded CHC/SPIO NBs; the same was not observed in the mouse that received the free dye (without incorporating with CHC/SPIO NBs). It is expected, in the future, both the dose of the therapeutic agent administered and its side effects can be significantly lowered by using novel CHC/SPIO NBs together with local delivery (intratumoral injection), targeted imaging and enhanced cellular uptake of the drug.

1. Introduction

In recent years, many drug delivery systems (DDSs) associated with imaging and targeting functions (i.e. image-guided DDSs) have been developed to detect diseases, deliver therapeutic agents and track the distribution of drug vehicles [1–4]. Image-guided DDSs can be probed via diagnostic imaging systems such as computed tomography, magnetic resonance (MR), fluorescence and ultrasound (US) [5–8]. An image-guided DDS combined with triggered release functions is expected to be helpful for the precise determination of the position and trigger timing of vehicles accumulated at a specific site. This can lead to the realization of the concept of the integration of diagnosis and therapy in a single box, which has been widely accepted and applied for the clinical treatment of many diseases [9–11].

Vehicles that perform both US imaging and US-triggered release functionalities have been extensively investigated because US is a noninvasive, radio-free and cost-effective form of energy that has long been employed in physical therapy, sonophoresis delivery and ultrasonic hyperthermia [8,12,13]. The most common US-triggered DDSs are microbubbles (MBs), which have been used to realize US intravascular images for monitoring the progression of angiogenesis and to deliver bioactive agents for antithrombus and gene therapies [12]. On the other hand, nanobubbles (NBs) have also attracted considerable attention in recent years because nanoscale objects circulating in blood capillaries can easily pass through the leaky vasculature in tumor tissue (having fenestration sizes of a few hundreds of nanometers, depending on the tumor type) to access the blood capillaries surrounding tumor cells (i.e. an enhanced permeation and retention (EPR) effect). In this light, NBs demonstrate a potential application for tumor imaging and

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drug delivery [13–16]. Generally, it is more difficult to prepare NBs than MBs owing to their thermodynamic instability. Many researchers have reported various materials and routes for preparing NBs. Wang et al. [17] used Tween and lipid-based molecules to prepare coumarin-6-loaded NBs as a US contrast agent. In addition, poly(lactic-co-glycolic acid) was employed to prepare dye-encapsulated NBs for US/optical dual-modal imaging [18]. Xing et al. [19] ultrasonicated a mixture of Span 60 and polyoxyethylene 40 stearate followed by differential centrifugation to obtain NBs. Several polymeric materials have been employed to prepare MBs and NBs [8,20,21]. Albumin- and lipid-based materials are the most commonly used bubble-forming materials for preparing bubble-based vehicles of various sizes. In the present study, we proposed a new bubble-forming material, carboxymethyl hexanoyl chitosan (CHC), to prepare multifunctional NBs and MBs by a facile sonication method without using surfactants or special equipment. CHC is an amphiphilic chitosan derivative bearing hydrophobic hexanoyl moieties and hydrophilic moieties that was developed by our group and has been employed as a biocompatible material for preparing micelles with high encapsulation efficiency for hydrophobic drugs [22,23]. Liang and Chiou et al. [24]employed CHC as a stem cell delivery system to enhance corneal wound healing. Ours is the first study to use CHC as a bubble-forming material to prepare image-guided NBs and MBs for the efficient encapsulation and delivery of hydrophobic drugs.

It remains difficult to acquire US images of NBs in a large animal by using a clinical US scanner (frequency: 5–15 MHz) because the US reflectivity is proportional to the fourth power of the bubble size and the signal strength is attenuated with the tissue depth [17]. Therefore, it is advantageous to use magnetic resonance imaging (MRI) as an alternative imaging method to probe NBs because the attenuation of MR signals is less significant than that of US signals in an animal body. In addition, MRI can provide structural and functional information at the molecular level [25]. Therefore, MBs with US/MR dual-modal imaging capability have been proposed by Kumacheva et al. and Gu et al. [26,27]. Plank et al. and Lammers et al. [28,29] developed MR image-guided MBs with US-triggered release behavior using lipid and poly(butyl cyanoacrylate) as bubble-forming materials, respectively. However, preparing NBs with MR imaging functionality, hydrophobic drug encapsulation and enhanced transcellular delivery still remains a challenge. This challenge was overcome in the present study by using a biocompatible bubble-forming material (CHC) and lipophilic superparamagnetic iron oxide (SPIO, an MR T_2 contrast agent) nanoparticles to prepare hydrophobic drug-loaded NBs, with the expectation that the images of bubbles can be probed by MRI and that transcellular drug delivery can be improved by the amphiphilic CHC and therapeutic US (frequency range: 1–3 MHz, power density: <3 W cm^{-2}). The CHC/SPIO-based bubble has not been reported thus far, and we believe that its imaging capability, drug encapsulation and drug delivery warrant further investigation.

The main objective of the present study was to use CHC as a new bubble-forming material to prepare novel image-guided bubbles (i.e. CHC/SPIO NBs for MR imaging and CHC/SPIO MBs for US imaging) for efficiently encapsulating and delivering hydrophobic agents. The effects of SPIO on the bubble size and drug encapsulation efficiency were studied. Subsequently, the effects of CHC/SPIO NBs on the in vitro CPT cytotoxicity under sonication and the in vivo tumor-specific accumulation after intratumoral injection were also investigated. An understanding of the ultrasonically activated behaviors of the superparamagnetic NBs and MBs could provide valuable fundamental information to support the design and fabrication of multifunctional image-guided drug carriers capable of intravascular imaging, extravascular imaging, magnetic target delivery, hyperthermia and remotely triggered release.

2. Materials and methods

Chitosan, camptothecin (CPT), Fe(acac)$_3$, 1,2-hexadecanediol, oleic acid (90%), oleylamine (70%), diphenyl ether (99%), hexanoic anhydride (97%), glucose (99%), and hexane were purchased from Sigma–Aldrich. CPT was employed as the model anticancer agent in this study because it demonstrated good stability under US sonication and cell culture conditions (i.e. fluorescence did not significantly alter upon US sonication). The animals used in the study were purchased from the Laboratory Animal Center of the National Yang-Ming University. All the animals were treated and housed following the protocol approved by the Institutional Animal Use and Care Committee of the National Yang-Ming University.

2.1. Preparation of CHC MBs, CHC/SPIO MBs and CHC/SPIO NBs

Lipophilic SPIO nanoparticles were prepared by following a method developed by Sun et al. [30]. Briefly, 2 mmol of Fe(acac)$_3$, 10 mmol of 1,2-hexadecanediol, 6 mmol of oleic acid and 6 mmol of oleylamine were dissolved in 20 ml of diphenyl ether and refluxed at 100 °C for 30 min under a nitrogen flow. Next, the mixture was heated to 200 °C for 1 h and then heated at 265 °C for 30 min. After centrifugation and washing, the SPIO nanoparticles were collected and redispersed into ethanol.

Our group has reported the synthesis of CHC using *N,O*-carboxymethyl chitosan (NOCC) as a precursor elsewhere [22,23]. NOCC samples (2 g) were dissolved in distilled water (50 ml) and stirred for 24 h. The resulting solutions were mixed with methanol (50 ml), following which hexanoic anhydride was added at a concentration of 0.5 M. After dialysis and drying, CHC was obtained. The mixture of CHC aqueous solution (1.5 wt.%) and glucose (3.3 mg ml^{-1}) was purged with SF_6 gas and sonicated using a probe-type sonicator (XL2000, Misonix Inc., USA) for 3 min. After sonication, the obtained milky suspension was placed in a refrigerator to allow the CHC MBs to separate by flotation. On the other hand, the CHC/SPIO MBs were obtained by mixing CHC aqueous solution (1.5 wt.%), glucose (3.3 mg ml^{-1}), SPIO (15–95 μg ml^{-1}) and hexane (2.5 vol.%). Finally, the CHC/SPIO NBs were obtained by mixing CHC aqueous solution (1.5 wt.%), glucose (3.3 mg ml^{-1}), SPIO (95 μg ml^{-1}) and hexane (0.3 vol.%). The CPT-loaded CHC/SPIO bubbles were obtained by adding 65 μl of CPT/ethanol solution (12 mg ml^{-1}) during the above preparation process. The process used subsequently for purge and sonication was the same as that in the case of CHC MBs. After sonication, the milky solution was then centrifuged (6000 rpm, 1 min) to collect bubbles, which were subsequently redispersed in distilled water. The obtained suspensions were freeze dried and stored in a vessel containing saturated SF_6 to eliminate hexane and to avoid early release. Before use, the dry bubble powders were restored by mixing with distilled water.

The amount of CPT encapsulated was determined by fully destroying an as-received CPT-loaded sample using low-frequency sonication (40 kHz). The hydrophobic CPT encapsulated was then extracted by a hexane solution in which the CPT concentration was determined using a multi-functional microplate reader (Tecan) to characterize the emission peak at 430 nm (excited at 370 nm) on the basis of a predetermined standard concentration–intensity calibration curve. The CPT payload in the CPT-loaded CHC/SPIO NB suspension for cell culture was determined as 70 μg ml^{-1}. Encapsulation efficiency (%) was calculated by the following equation:

$$\text{encapsulation efficiency } (\%) = (\text{amount of CPT encapsulated}/\text{amount of CPT added in bubble preparation process}) \times 100\%$$