Ultrasonics Sonochemistry 37 (2017) 235–243

Contents lists available at ScienceDirect

Ultrasonics Sonochemistry

journal homepage: www.elsevier.com/locate/ultson

Development of a novel folate-modified nanobubbles with improved targeting ability to tumor cells

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ARTICLE INFO

Article history:
Received 9 December 2016
Received in revised form 9 January 2017
Accepted 9 January 2017
Available online 10 January 2017

Keywords:
Nanobubble
Folate receptor
Ultrasound molecular imaging
Targeted theranostic
Tumor

ABSTRACT

Conjugation of folate (FOL) to nanobubbles could enhance the selective targeting to tumors expressing high levels of folate receptor (FR). To further improve the selective targeting ability of FOL-modified nanobubbles, a novel FOL-targeted nanobubble ($(FOL)_2$-NB) with increasing FOL content (accomplished by linking two FOL molecules per DSPE-PEG2000 chain) was synthesized, through the methods of mechanical shaking and low-speed centrifugation based on lipid-stabilized perfluoropropane. The bubble size and distribution range were measured by dynamic light scattering (DLS). Enhanced imaging ability was evaluated using a custom-made agarose mold with a clinical US imaging system at mechanical indices of up to 0.12 at a center frequency of 9.0 MHz. Targeted ability was also carried out in human breast cancer MCF-7 cells, which over-express the FR, by fluorescence activated cell sorting (FACS) and fluorescence microscopy, respectively. $(FOL)_2$-NB with a particle size of 286.87 ± 22.96 nm were successfully prepared, and they exhibited superior contrast imaging effect. FACS and fluorescence microscopy studies showed greater cellular targeting ability in the group of $(FOL)_2$-NB than in their control group of Non-targeted-NB (no FOL targeted nanobubbles) and FOL-NB (one FOL molecule per DSPE-PEG2000 chain). These results suggest that a new type of stronger targeted nanobubble was successfully prepared by increasing the FOL content per DSPE-PEG2000 chain. This novel $(FOL)_2$-NBs are potentially useful for ultrasound molecular imaging and treatment of FR-positive tumors and are worthy for further investigation.

1. Introduction

Traditional chemotherapy therapeutic strategies have several limitations including lack of sufficient efficacy, innate and acquired drug resistance, as well as serious untoward toxicity. Several interesting current strategies for overcoming these limitations including: encapsulating of drugs in a vehicle, such as ultrasound contrast agent (UCA) [1]; selective delivering of drugs to tumor sites with targeted theranostic agents [2]. UCA has enhanced the capabilities of ultrasound as a molecular imaging modality and stimulated innovative strategies for cancer theranostic during the past two decades. The UCA was divided into two categories of microbubble and nanobubble according to their size. As a kind of emerging UCA with sizes less than 1 μm, nanobubble are most investigated in recent time. Nanobubble has attracted considerable research attention due to their priorities in ultrasound molecular imaging and potential therapy such as a passive targeting mechanism known as enhanced permeability and retention effects (EPR) [3]. The smaller size of nanobubble can overcome the limitations of microbubble, therefore, they can pass through the vessel wall into the tumor tissue, and can be applied to tumor-targeted imaging and therapy because the vascular endothelial gap in tumors is approximately 380–780 nm, which is much wider than that of normal tissue with a vascular endothelial gap of less than 7 nm [4].

In addition to the EPR effects, active tumor cell targeting is also a promising strategy for therapeutic enhancement of chemotherapeutic agents by ultrasonic nanobubble. The development of targeted nanobubble has been gaining interest in the field of tumor-targeted treatment and controlled drug delivery thanks to their ability to carry and release drugs under monitoring by ultrasound imaging. Nanobubble could be surface modified to be targeted bubble [5], and the most common strategies for site targeting of bubbles are principally to attach disease-specific ligands, such as antibodies, aptamers and peptides [6–9]. The FR, one of the most exploited targets, is usually over-expressed in many malignant tumors such as breast, kidney, ovary, uterus, colon and lung [10–12]. In normal tissues, FR are present either in low or non-detectable quantities or in areas not accessible by flood flow

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http://dx.doi.org/10.1016/j.ultsonch.2017.01.013
1350-4177/

[11]. These features have encouraged the development of FR-targeted strategies for tumor diagnostics and treatments. In recent years, a number of FOL conjugated therapeutic and imaging agents have been evaluated in preclinical studies of liposomal agents [13–17] or UCAs [18–21], with encouraging results. Thus, FR is viewed as a therapeutic target that may provide an effective option for targeted personalized tumor theranostic.

However, nearly all nanobubbles or microbubbles in previous studies were prepared based on DSPE-PEG2000 polymer chain which only can carry one FOL residue at a time, because it has only one carboxyl terminal. Suppose one DSPE-PEG2000 chain could carry two or more FOL molecules, the targeted nanobubbles maybe more effective in selectively targeting FR-positive tumor cells. Therefore, to further improve the selective targeting ability of nanobubbles, a novel targeted nanobubble ($(FOL)_2$-NB) with two FOL molecules per DSPE-PEG2000 chain was synthesized. Increasing FOL content per DSPE-PEG2000 chain was accomplished with the help of L-2-aminoadipic acid (AD), as illustrated in Fig. 1.

On the basis of these considerations, the aims of this study were: (i) to prepare twofold FOL targeted nanobubble ($(FOL)_2$-NB), and (ii) to investigate the selective targeting ability of $(FOL)_2$-NBs to FR-positive tumor cells in vitro. The bubble size and distribution range were measured by DLS method. Enhanced imaging ability was evaluated using a custom-made agarose mold with a clinical US imaging system at mechanical indices of up to 0.12 at a center frequency of 9.0 MHz. Targeted ability was also carried out in human breast cancer MCF-7 cells, which overexpress the FR, by fluorescence activated cell sorting (FACS) and fluorescence microscopy, respectively. To properly study the activity of $(FOL)_2$-NBs, analogous carboxyl-terminated nanobubble (Non-targeted-NB) without FOL incorporation and another targeted nanobubble (FOL-NB) with one FOL molecule per DSPE-PEG2000 chain were studied as control.

2. Materials and methods

2.1. Chemicals

The formation of acoustic nanobubble comprised perfluoropropane (C_3F_8) as the core and a coating of lipid mixture as the stabilizing shell. The C_3F_8 was purchased from R&D Center for Specialty Gases at the Research Institute of Physical and Chemical Engineering of Nuclear Industry (Beijing, China). The lipid components including 1,2-distearoyl-*sn*-glycero-3-Phosphoethanolamine (DSPE), dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), were purchased from Sigma-Aldrich (MO, USA). The folate, L-2-aminoadipic acid (AD), N-Boc-1,5-Diaminopentane (BOC-DAP), heterobifunctional poly(ethylene glycol) (HO-PEG-COOH), N-Hydroxysuccinimide (NHS) and Diethyl 1,4-dihydro-2,4,6-trime thyl-3,5-pyridinedicarboxylate (DDC), were also purchased from Sigma-Aldrich (MO, USA). The phosphate-buffered saline (PBS, pH 7.4), fluorescent probes Dil and DAPI were purchased from Beyotime (Haimen, China). The agar powder for fabricating gel phantoms for the in vitro experiments was purchased from Invitrogen (CA, USA). The DSPE-PEG2000-FOL was from Avanti polar lipids (USA).

2.2. Cell line

The MCF-7 human breast carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD). DMEM medium, 0.25% trypsin-EDTA and heat-inactivated fetal bovine serum was from Gibco BRL Life Technologies (Paisley, UK). Cell counting kit-8 (CCK-8) was from Sigma-Aldrich (MO, USA).

2.3. Synthesis of DSPE-PEG2000-AD-$(FOL)_2$

With reference to the literature [22], the synthesis route of DSPE-PEG2000-AD-$(FOL)_2$ was proposed with some modifications, which is described as follows:

DSPE-PEG2000-NHS: DSPE-PEG2000-COOH (280 mg) was dissolved in CH_2Cl_2 (8 mL), followed by adding NHS (17.5 mg) and DCC (62 mg). The mixture was reacted at room temperature in the dark for 3 h under stirring. After the completion of the reaction, the mixture was filtered to remove DCU. Then, cold diethyl ether (20 mL) was added into the solution, the mixture was reacted at −20 °C for 4 h. Finally, the DSPE-PEG2000-NHS was recovered by filtration and dried under vacuum.

DSPE-PEG-AD-$(COOH)_2$: AD was dissolved in borate buffer (10 mL, 0.1 M, pH = 8.0), followed by adding DSPE-PEG2000-NHS (300 mg), the mixture was reacted in the dark under stirring for 0.5 h. HCl (0.2 N) was added and adjusted the pH to about 4–4.5, then $CHCl_3$ (3 × 80 mL) was added in order to purify the product from the excess of AD. The organic phase, dried over anhydrous Na_2SO_4, was concentrated under vacuum, and cold diethyl ether (200 mL) was added under stirring. The mixture was reacted at −20 °C for 4 h, finally, the *DSPE-PEG-AD-$(COOH)_2$* was filtered and dried under vacuum.

FOL-NH_2: FOL (441 mg) was dissolved in anhydrous DMSO (20 mL) at 40 °C under stirring, followed by adding BOC-DAP (428 mg) and DCC (572 mg). The mixture was reacted for 4 h and then dropped into cold diethyl ether (300 mL), after 5 h at −20 °C, the precipitate was filtered, washed with THF and dried under vacuum. Finally, intermediate FOL-DAP-BOC (654 mg) was dissolved in anhydrous CH_2Cl_2 and followed by adding cold TFA (1 mL), the mixture was reacted at room temperature for 2 h. Vacuum evaporation method was adapted to remove excess TFA and CH_2Cl_2, cold diethyl ether (50 mL) was added under stirring, and the products was filtered and dried under vacuum.

DSPE-PEG2000-AD-$(FOL)_2$: DSPE-PEG-AD-$(COOH)_2$ (300 mg) was dissolved in anhydrous DMF (5 mL), followed by adding FOL-DAP-NH_2 (55 mg) dissolved in anhydrous DMSO (1 mL) drop wise under stirring. The mixture was reacted at room temperature in the dark for overnight. Then, dH_2O (8 mL), HCl (0.1 N, 6 mL) and NaCl saturated solution (2 mL) were added, chloroform (4 × 40 mL) was used to purify the product. After dried over anhydrous Na_2SO_4, the organic phase was concentrated under vacuum. Finally, cold diethyl ether (50 mL) was added under stirring. The mixture was reacted at −20 °C for 3 h, and the *DSPE-PEG2000-AD-$(FOL)_2$* was recovered by centrifugation at 10 °C at 1500*g* for 10 min and dried under vacuum.

2.4. Nanobubbles preparation

Fixed-ratio mixture of the phospholipids including DSPE-PEG2000-AD-$(FOL)_2$, DPPC, Span-60 and Tween-80 were added to a 1.5 mL EP tube and dissolved in 1.0 mL of PBS. The air in the tube was replaced with C3F8 gas using a 10 mL syringe with a long, fine needle. Until the mixture was completely dissolved with water bath of 60 °C, then a small amount of the fluorescent membrane probe Dil (red fluorescence) was added. The air in the tube was replaced with C3F8 gas again. Finally, the tube was oscillated for 90 s in a mechanical oscillator (Ag and Hg mixer, Xi'an, China). The mixture was diluted to PBS (5 mL), and then collected the low layer of the solution after centrifuging for 5 min at 300 rpm. The Non-targeted-NB and FOL-NB were synthesized using the same procedure. All tubes were covered with aluminum foil to prevent fluorescence quenching.