Ultrasonics 99 (2019) 105947

Contents lists available at ScienceDirect

Ultrasonics

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

Development of novel ST68/PLA-PEG stabilized ultrasound nanobubbles for potential tumor imaging and theranostic

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ARTICLE INFO

Keywords:
Nanobubble
Ultrasound
Nanomedicine
Ultrasound contrast agents

ABSTRACT

Nanobubbles (NBs) have received wide attention as theranostic agents and been extensively explored in various applications, especially in cancer. The aim of this study was to develop a novel kind of NBs which possess high echogenicity and good stability. This novel ultrasonic nanobubbles (ST68/PLA-PEG NBs) consist of perfluoropropane gas stabilized by Span 60 and Tween 80 (ST68) surfactant and synthesized PLA-PEG-NH_2 block copolymers, and were prepared through the methods of mechanical shaking and low-speed centrifugation. A series of experiments were carried out to evaluate the physicochemical properties, echogenicity and cytotoxicity of this novel NBs. According to the amount ratio of copolymers to surfactant, the NBs were divided into 5 groups (0%, 5%, 10%, 15% and 20%). Group "10%" were the optimum NBs, with a size of 675.6 nm, polydispersity index of 0.39. Moreover, these NBs gave a maximum contrast intensity of 31.0 ± 0.2 dB over baseline and little loss of contrast signal after 10 min. In conclusion, this novel kind of ST68/PLA-PEG NBs which exhibited a high echogenicity and good stability were successfully prepared, and they may offer a potential strategy for drug delivery and tumor-targeted theranostic.

1. Introduction

With the advent and advancement in ultrasound contrast agents (UCAs), the capabilities of ultrasound have been enhanced as a molecular imaging modality and stimulated innovative strategies for ultrasound-mediated drug and gene delivery in recent decades [1,2]. UCAs have received wide attention as theranostic agents and have been extensively explored in various applications, especially in cancer management [2–4]. UCAs could be divided into two categories of microbubbles (MBs) and nanobubbles (NBs) according to their sizes. Differ from MBs, NBs have enormous potential in tumor-targeted drug delivery and theranostic because they can pave the way for extravasation from blood vessels into surrounding tissues, thus improving delivery efficiency and localization [5].

The most promising NBs developed so far are generally composed of poorly water soluble gas (such as perfluoropropane and sulfur hexafluoride) stabilized by different kinds of shells, such as surfactant [6,7], lipid [8,9], polymer [3,4], or other proprietary shell materials. NBs prepared by different shell materials have their own advantages and drawbacks in ultrasound-mediated imaging and drug/gene delivery. In details, surfactant NBs have strong echogenicity, yet are very unstable. Lipid NBs are with strong echogenicity and easy penetrability but are limited by the poor stability. Polymer NBs are stable enough and easy to be modified by various functional moieties and have controlled drug release profiles, but have very weak echogenicity. From the knowledge mentioned above, we can conclude that the shell materials play a crucial role in the characterization and theranostic performance of NBs.

Differ from the NBs reported previous, in this study, we aimed to develop a novel kind of NBs which consist of PFC gas stabilized by Span 60 and Tween 80 (ST68) surfactant and synthesized PLA-PEG-NH_2 block copolymers. The resulting NBs were expected to simultaneously have the performance of high stability, high echogenicity, and potential target capacity and controlled drug release profiles. To the best of our knowledge, incorporating PLA-PEG-NH_2 block copolymers to surfactant in nanobubble preparation is a debut report.

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https://doi.org/10.1016/j.ultras.2019.105947
Received 9 May 2019; Received in revised form 7 June 2019; Accepted 19 June 2019
Available online 02 July 2019
0041-624X/

2. Materials and methods

2.1. Materials

Ethylene oxide (EO) (Shanghai, China) was distilled over calcium hydride. D,L-lactide (DL-LA) (Jinan, China) was recrystallized from dry ethyl acetate for three times. Tetrahydrofuran (THF) (Shanghai, China) was refluxed in the presence of metal sodium under argon. Potassium bis (trimethylsilyl) amide ($[(CH_3)_3Si]_2NK$) (0.5 mmol/mL) was purchased from Fine Chemical Reagent Co, Ltd (Tianji, China). Phosphate-buffered saline (PBS, pH 7.4) was purchased from Beyotime (Haimen, China). Span 60 (sorbitan monostearate) and Tween 80 (polyoxyethylene-sorbitan monooleate) were purchased from Sigma-Aldrich (MO, USA). PFC gas (octafluoropropane, 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). All other chemicals were of analytical reagent or higher grade.

2.2. Synthesis of PLA-PEG-NH_2 block copolymers

PLA-PEG-NH_2 block copolymers were synthesized by a single one step anionic ring-opening polymerization method, which is described briefly as follows: to a dried ampoule filled with argon, 5 mL EO, 4.35 mL$[(CH_3)_3Si]_2NK$, and 20 mL THF were injected at 0 ℃. After stirring for 24 h at 40 ℃, 6.525 g DL-LA was added and stirred for 1.5 h at a room temperature. Then 2 mL methanol was added and the reaction was carried out at a room temperature for 30 min. The living polymerization reaction was terminated by adding 10 mL HCl and stirring for 15 min. The viscous polymer solution was precipitated in ether/methanol (v/v = 3:1). The resultant powder was collected after drying under vacuum for 48 h.

Molecular weight and molecular weight distribution of the synthesized PLA-PEG-NH_2 block copolymers were assessed by the gel permeation chromatography (GPC) system (Waters 510 chromatographic instrument, USA) equipped with a refractive index detector. THF was used as an eluting solvent. The GPC data were calibrated with polystyrene with different molecular weights as the standards. The chemical structure of the synthesized PLA-PEG-NH_2 block copolymers was confirmed by the Fourier transform infrared spectrum (FTIR) (AXIS Ultra DLD, Kraotos Instruments Corporation, UK) and ^1H NMR spectroscopy (AVANCE III-400 MHz, Bruker, Karlsruhe, Germany) using DSMO solvent.

2.3. Preparation of ST68 solution

ST68 solution was prepared following the steps of previously reported method by Oeffinger et al. [6]. Briefly, an intimate mixture of Span 60 (1.48 g) and Tween 80 (1.00 mL) was made by suspending these components with sodium chloride (NACl) (1.50 g) in PBS (50 mL). The mixture was stirred at 60 ℃ for 15 min and heated to a temperature of 100 ℃ and held there for 15 min, then cooled to room temperature before autoclaving (Tuttnauer Brinkmann 3850e, Westbury, NY, USA) using a lipid cycle for 12 min at 120 ℃ at atmospheric pressure. Finally, the resulting ST68 solution was in the form of white emulsion.

2.4. Preparation of NBs

A certain amount of PLA-PEG-NH_2 block copolymers were added to the ST68 solution (1 mL) in 1.5-mL EP tube. The mixture was heated to 65 ℃ to make PLA-PEG-NH_2 block copolymers completely dissolved and then cooled to room temperature. Then the air in the tube was purged with PFC gas using a 10-mL syringe with a long, fine needle and then the tube was tightly capped. Finally, the tubes was oscillated for 60 s in a mechanical oscillator (Ag and Hg mixer, Xi'an, China) to generate the bubbles (ST68/PLA-PEG stabilized bubbles). The mixture in the tube was held there for 10–15 min. Then, the solution separated into two distinct layers: the upper layer containing mostly foam, and the lower layer containing suspended bubbles in buffer. The lower layer of the solution was collected, and the top layer was discarded. The collected solution was diluted to 3 mL with PBS. After dilution, the collected solution was centrifuged for 3 min at 300 r/min. Finally, the intermediate layer mostly containing NBs was separated from the upper layer and the lower layer.

To optimize the materials ratio, the NB samples were divided into 5 groups (0%, 5%, 10%, 15% and 20%, respectively) according to the amount ratio of PLA-PEG-NH_2 block copolymers to ST68 surfactant. Among them, NBs of Group "0%" were prepared with pure ST68 surfactant using the same procedure and were used as control. What needs to be mentioned is that the amount ratios were set according to our pre-experiment results.

2.5. Morphology and size analysis

The NB sample was diluted fivefold, and 10 μL of the diluted suspension was then dropped onto a slide, which was covered with a coverslip. The morphology and distribution of the NBs were then observed under a microscope at 400× and 1000× magnifications (OLYMPUS BX41, Olympus Corporation, Tokyo, Japan). The bubble size, distribution range and polydispersity index (PDI) were measured with a Zetasizer Nano ZS90 analyzer (Malvern Instruments Ltd., Worcestershire, UK). The number of NBs was counted by a hemacytometer. All measurements were carried out three times and averaged. All experiments were conducted in triplicate.

2.6. Echogenicity determination in vitro

In order to assess the echogenicity of the NB samples, an *in vitro* acoustic test was performed by exposing the NBs to a medical ultrasound beam as shown in Fig. 6a. Briefly, 0.4 mL NBs sample at concentration of 1.0×10^6 bubbles/mL was added to 10 mL degassed PBS in a condom submerged in a degassed, deionized water tank. The ultrasound contrast enhancements were monitored by a medical ultrasound scanner (LOGIQ E9; GE, USA) system in contrast mode for 0 min to 10 min with the following parameters: focal length, 3.0 cm; transmit power, 28%; mechanical index, 0.12; dynamic range, 60 dB and a center frequency of 9.0 MHz. The ML6-15 transducer was inserted into the degassed, deionized water and focused through the NBs sample. Ten images of each sample at different time points were acquired. Time-response intensity curves were recorded. The contrast intensity was analyzed using "Q analysis" software of the scanner. Circular regions of interest (ROIs) were outlined in each sample well. The contrast intensity of the NBs samples were normalized to that of the degassed, deionized water. Survival ratio was calculated as the contrast intensity at each time point/the maximum contrast intensity × 100%. All measurements were carried out three times and averaged. All experiments were conducted in triplicate.

2.7. Cytotoxicity assay

CCK-8 assay was performed to evaluate cell viability. MCF-7 cells were seeded in 96-well plates at a density of 5×10^3 cells/well and then grow in culture medium (100 μL of DMEM medium containing 10% fetal bovine serum) in a humidified atmosphere with 5% CO_2. After incubation for 24 h, the culture medium was then replaced with a same volume of fresh medium containing the optimum NBs samples at different concentrations. After incubation for another 24 h, the medium containing the optimum NBs samples was discard and replaced with fresh medium (100 μL) containing 10 μL of CCK-8 solution. And the cells were subsequently cultured for 4 h. After gentle agitation for 5 min, an Infinite F200 multimode plate reader (Tecan, Männedorf, Switzerland) was used to detect the absorbance of each well at 450 nm. All experiments were conducted in triplicate.

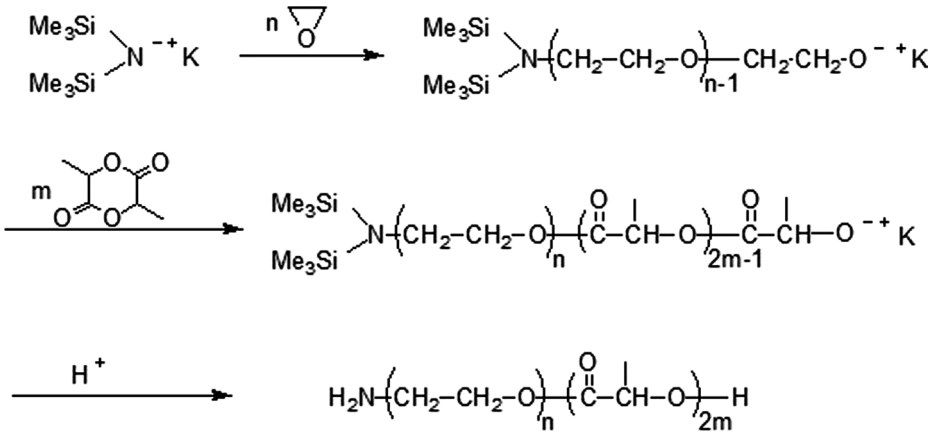

Fig. 1. Synthetic route of PLA-PEG-NH_2 block copolymers.

2.8. Statistical analysis

Statistical significance of differences between different groups was derived using one-way ANOVA. Unpaired Student's *t* test was performed for the comparisons of contrast intensity. The data was statistically analyzed with SPSS software (version 13.0; SPSS Inc, Chicago, IL). A P value < 0.05 was considered statistically significant. All statistical tests were two-tailed.

3. Results

3.1. Synthesis and characterization of PLA-PEG-NH_2 block copolymers

The synthesis pathway of PLA-PEG-NH_2 block copolymers was illustrated in Fig. 1. Fig. 2a showed the GPC curves of PEG and PLA-PEG-NH_2 block copolymers, respectively. Compared with PEG (Mn = 2000), the peak of PLA-PEG-NH_2 (Mn = 5000) was shifted to left, indicating the formation of high molecular weight block copolymers. In addition, the curve representing the resulting PLA-PEG-NH_2 block copolymers was with single peak and narrow molecular weight distribution.

The composition of PLA-PEG-NH_2 block copolymers was determined from the ^{1}H NMR spectrum (Fig. 2b). The characteristic resonances corresponding to PEG and PLA units are observed clearly at 3.64, 5.29, and 1.56 ppm, respectively. The resonance at 3.64 ppm came from the EO protons. The resonance at 5.29 ppm was from the D,L-LA protons. The large resonance peak at 1.56 ppm was attributed to the protons of the lactic repeat units of PLA-PEG-NH_2 block copolymers. The resonance of –NH_2 was not observed. This was due to its low content in the high molecular weight block copolymers and hydrogen bonding interactions.

The FTIR spectrum of PLA-PEG-NH_2 block copolymers was shown in Fig. 2c. The absorption peak at 1092 cm^{-1} was attributed to C—O—C stretch of PEG segments. The strong absorption peak at 1756 cm^{-1} belonged to —C═O stretch, indicating the formation of PLA segments. The broad absorption peak at 3243 cm^{-1} was —OH stretching, which was practically eliminated from the spectrum of PLA-PEG block copolymers.

All these results above demonstrated that the required PLA-PEG-NH_2 block copolymers were successfully synthesized.

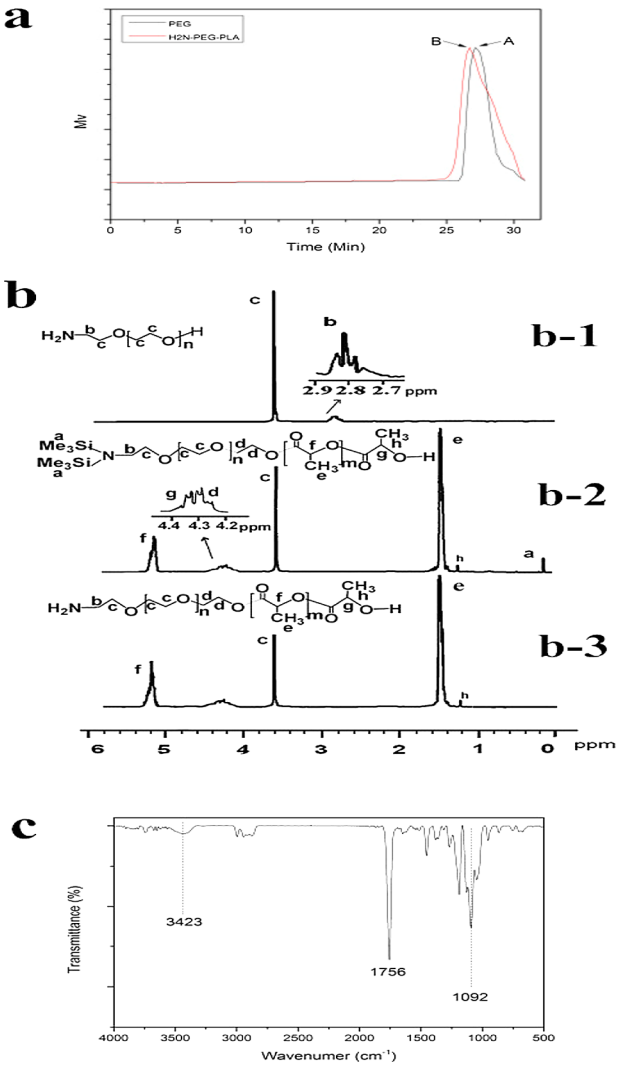

Fig. 2. Characterization of PLA-PEG-NH_2 block copolymers by (a) GPC; (b) NMR; (c) FTIR.

3.2. Preparation and characterization of NBs

The novel ST68/PLA-PEG stabilized NBs were successfully prepared. The schematic diagram was shown in Fig. 3. Fig. 4 showed the microscopy images of the resulting ST68 stabilized NBs (Fig. 4a) and ST68/PLA-PEG stabilized NBs (Fig. 4b). The typical size distribution of the NBs, as measured with a Zetasizer Nano analyzer, was presented in Fig. 5. The size increased nonlinearly with the amount ratio of PLA-PEG-NH_2 block copolymers to ST68 surfactant increased. The size distributions of three groups (Group "0%", "5%", "10%") were within our

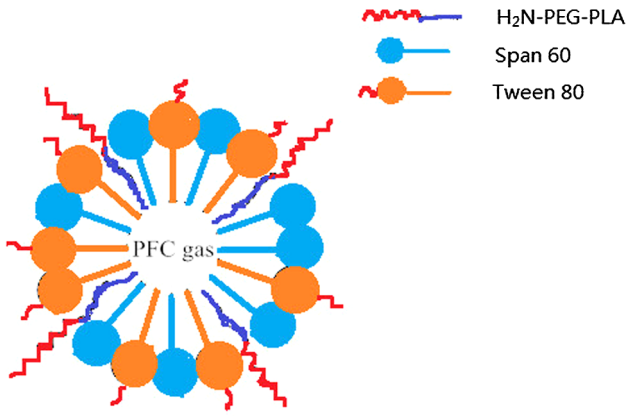

Fig. 3. Schematic diagram of ST68/PLA-PEG stabilized NBs.

specified requirements (< 780 nm) and were expected to undergo passive targeting to tumor cells through EPR effect. Unfortunately, NBs of Group "15%" and Group "20%" were not within our specified requirements.

3.3. Echogenicity of NBs as UCAs for US imaging

The relative acoustic contrast data at 9.0 MHz *in vitro* was shown in Fig. 6b. The maximum contrast intensity immediately after addition of different NBs samples were 29.6 ± 0.3 dB (0%), 29.2 ± 0.7 dB (5%), 31.0 ± 0.2 dB (10%), 29.4 ± 0.5 dB (15%) and 25.2 ± 0.7 dB (20%), respectively. The contrast intensity of Group "10%" was higher than those of the other groups ($P < 0.05$). While the contrast intensity of Group "20%" was lower than those of the other groups ($P < 0.05$). The contrast intensity showed a trend of first increase and then decrease when increasing the amount ratio of PLA-PEG-NH_2 block copolymers to ST68 surfactant.

The ultrasound contrast intensity was continuously monitored for 10 min to analyze the time response contrast signal loss after addition of NBs. The contrast intensities from 0 min to 10 min were plotted as a time course (Fig. 6c) and the survival ratios were calculated (Table 1). As seen, the contrast intensities at each time point of Group "10%" were significantly higher than those of the other groups. The decay of ST68/PLA-PEG stabilized NBs was steady, while ST68 stabilized NBs had an obvious decay from 0 min to 1 min. The survival ratios of ST68/PLA-PEG stabilized NBs were larger than that of ST68 stabilized NBs at all ten time points. The survival ratio of different groups approached 50% in the time period of: > 10 min (10%), 9–10 min (15%), 8–9 min (5%), 6–7 min (20%) and 3–4 min (0%).

3.4. Optimization of ST68/PLA-PEG stabilized NBs

On the basis of the above results, NBs of Group "10%" was the optimum one, under this condition, the amount ratio of PLA-PEG-NH_2 block copolymers to ST68 surfactant was 10% for preparation. This optimum ST68/PLA-PEG stabilized NBs were with a size of 675.6 nm, PDI of 0.39. Moreover, these optimum NBs gave a maximum contrast intensity of 31.0 ± 0.2 dB over baseline and little loss of contrast signal was observed over 10 min duration of analysis (survival ratio, 54.84%), much longer than the time would be needed for diagnostic imaging practice (Fig. 7).

3.5. Cytotoxicity assays of NBs

The cytotoxicities of the optimum NBs were evaluated by incubating the cells with the optimum NBs samples with concentrations from 0 to 1000 μg/mL using the CCK-8 assay. Fig. 8 showed the cell viability of the MCF-7 cell line was higher than 90%, even at very high concentration of the optimum NBs samples (1000 μg/mL) after 24 h incubation, demonstrating that the optimum NBs are of low cytotoxicity and of great potential when further used *in vivo*.

4. Discussion

The emergence of theranostics with ultrasound technology is a promising development, as they open pathways to provide more effective treatments [10], especially for cancer. Compared with MBs, NBs may potentially be better ultrasound theranostic agents as their nanoscaled sizes. The ultimate aim of this study was to develop a novel kind of NBs with favorable ultrasound characteristics and improved performance. To reach this aim, a novel ST68/PLA-PEG stabilized NBs were successfully prepared by incorporating PLA-PEG-NH_2 block copolymers into ST68 surfactant. The integration of surfactant and copolymers was in lights of pros and cons of these two different shell materials. The resulting optimum NBs (the amount ratio, 10%) in this study were characterized to be with a spherical morphology and size of 675.6 nm. NBs with this size could extravasate from the leaky pores (380–780 nm) of vessels [11] into tumor parenchyma via EPR effect.

The echogenicity of the novel ST68/PLA-PEG stabilized NBs was assessed *in vitro* and the results were encouraging. The contrast intensity showed a trend of first increase and then decrease when increasing the amount ratio of PLA-PEG-NH_2 block copolymers to ST68 surfactant. As PLA-PEG-NH_2 block copolymers are with long and flexible chains, when the amount ratio is low, the copolymers could interact sufficiently with ST68 surfactant to self-assemble to form a flexible bubble shell. Then the improved shell flexibility may contribute to a higher echogenicity. Nevertheless, the emulsifying capacity of the

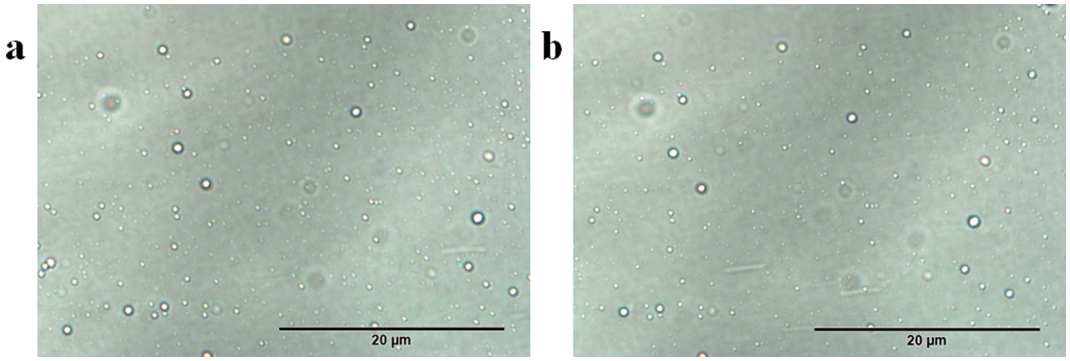

Fig. 4. Microscopy image of (a) ST68 stabilized NBS and (b) ST68/PLA-PEG stabilized NBs (1000×).

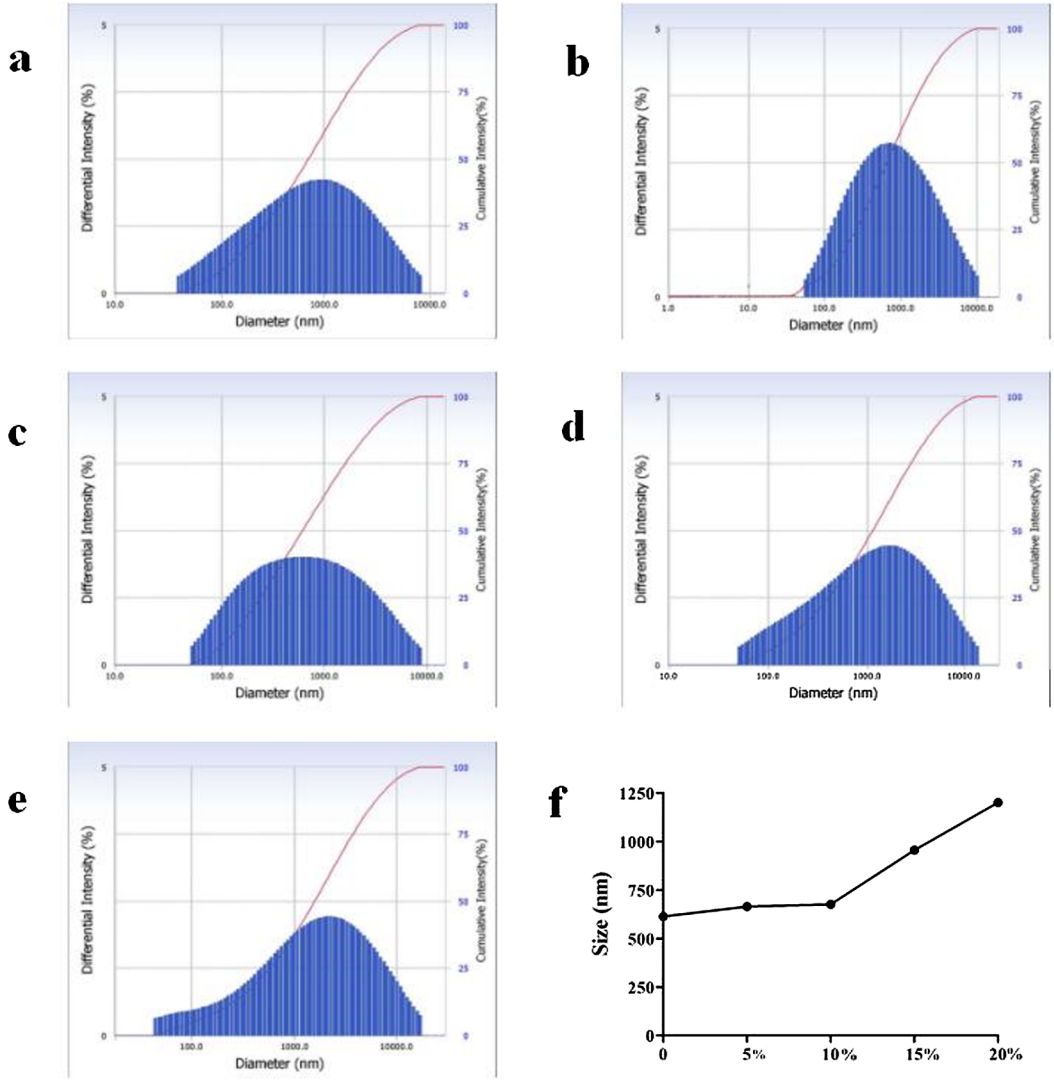

Fig. 5. Size distribution of NBs obtained by a Zetasizer Nano analyzer (a) Group "0%"; (b) Group "5%"; (c) Group "10%"; (d) Group "15%"; (e) Group "20%"; (f) Comparison of sizes among the five groups.

surfactant would drop as the amount ratio increases. When the amount ratio is too high, copolymers may have a strong potential to leave the shell due to the entrapment by other copolymers and then self-assemble into polymer bubbles, which are difficult to be detected by a medical ultrasound scanner. This point could be used to explain that the maximum contrast intensity of Group "20%" was lower than those of the other groups ($P < 0.05$) and the bubble size increased nonlinearly with the amount ratio increased. When the amount ratio increases to a certain value, the copolymers may not be well emulsified and then deposit. This phenomenon ever appeared in our pre-experiments of NBs with the amount ratio of 40%. What's more, the decay of the ST68/PLA-PEG stabilized NBs was steady, while the ST68 stabilized NBs had an obvious decay from 0 min to 1 min (Fig. 6c). The time when survival ratio approaching 50% was obviously prolonged by incorporating PLA-PEG-NH_2 block copolymers, with an descending order of Group "10%", "15%", "5%", "20%" and "0%" (Table 1). This phenomenon may be attributed to that PEG molecules can interact with ST68 surfactant to form a low surface tension bubble shell. The reduction in surface tension could effectively improve the stability of the bubbles [7,12,13].

PLA-PEG-NH_2 block copolymers were synthesized by the ring-opening polymerization method with some modifications in this study. In most of the previous reports about LA ring-opening polymerization, stannous octanoate [$Sn(Oct)_2$] catalyst has been widely used [14]. However, its cytotoxicity has recently caused deep concern, particularly when being used for biomedical purposes. While here the potassium-based initiator—$[(CH_3)_3Si]_2NK$ was used as an alternative of catalyst. The use of nontoxic potassium alcoholate initiators in this study was not only safe [15] for biomedical applications, but also simplified the reaction steps. Safety, in combination with its low cost, defines ST68/PLA-PEG-NH_2 as the preferred shell material for subsequent bioimaging and therapeutic delivery system. Besides PLA-PEG-NH_2, the other material such as Span 60 [16], Tween 80 [17] and PEG used in this study have been approved by FDA and are known to be with attractive properties of being biocompatible, biodegradable and nontoxic for clinical applications. However, the cytotoxicity experiments with CCK-8 kit were also carried out to evaluate their biological safety. As expected, the results in Fig. 8 showed that, at commonly used concentration, there was nearly no toxicity to cells, which indicate that the materials used for preparing NBs in this study were indeed safe enough.

Easy modification and controlled drug release are also important

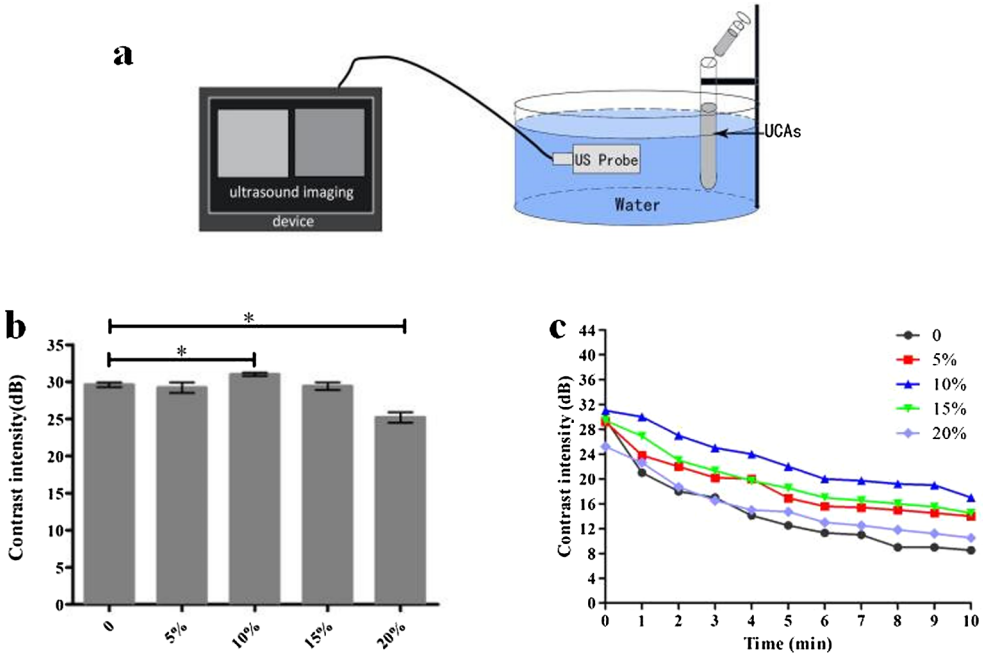

Fig. 6. Capacity as ultrasound contrast agents for enhancement imaging *in vitro*. (a) Set-up to monitor the ultrasound contrast intensity; (b) Maximum contrast intensity of NBs (*$P < 0.05$); (c) Time-intensity curve of NBs.

criterions for ultrasound NBs. Although relative experiments have not yet been done, this novel ST68/PLA-PEG stabilized NBs are very likely meeting the above two functions. Additional $–NH_2$ groups with high reactivity were integrated in the novel NBs shells make them to be easily modified by various functional ligands, such as antibody, protein, peptide or small molecules [18]. Acting as a controlled drug delivery system is one of the most important characteristics of copolymer-based NBs. As PLA-PEG-NH_2 block copolymers were integrated in the novel NBs shells in this study, the novel NBs could be expected with the potential for controlled drug release profiles.

Given the above, this novel ST68/PLA-PEG stabilized NBs may hold great promise because of their advantages such as high echogenicity, steady decay profile, safety, low cost, easy modification and potential controlled drug release. However, future studies are still warranted to elucidate the possible theranostic benefits using this novel nanobubbles, including *in vivo* contrast enhancements, surface modifications by functional ligands and drug delivery capacities.

5. Conclusions

A novel ST68/PLA-PEG stabilized NBs were successfully prepared by incorporating PLA-PEG-NH_2 block copolymers into ST68 surfactant. The resulting optimum NBs were with a required size less than 780 nm. The novel NBs exhibited a superior performance in stability, echogenicity, decay profile and low cytotoxicity. Moreover, this novel NBs are expected to be modified by various functional ligands to gain

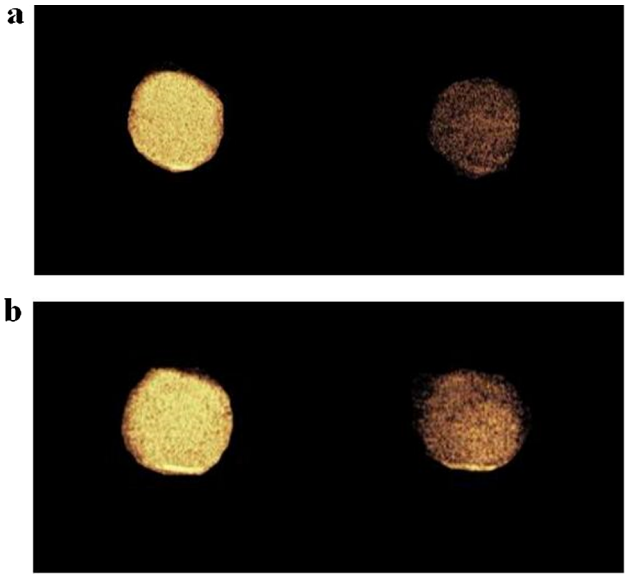

Fig. 7. The initial phase (0 min) and terminal phase (10 min) of ultrasound contrast images (a) Group "0%": The contrast intensity was 29.6 ± 0.3 dB at initial, the survival ratio was only 28.72% after 10 min; (b) Group "10%": The contrast intensity was 31.0 ± 0.2 dB at initial, the survival ratio was up to 54.84% after 10 min.

Table 1
Survival ratio (%) of NBs at different time points.

Group	1 min	2 min	3 min	4 min	5 min	6 min	7 min	8 min	9 min	10 min
0%	70.95	60.81	57.43	47.64	42.23	38.18	37.16	32.43	30.41	28.72
5%	81.51	75.34	69.18	68.49	57.88	53.42	52.74	51.37	49.66	47.95
10%	96.77	87.10	80.65	77.42	70.97	64.52	63.55	61.94	61.29	54.84
15%	91.50	78.23	72.45	67.01	62.93	57.82	56.12	54.42	52.72	49.32
20%	89.68	74.21	65.48	59.52	58.33	51.59	49.60	46.83	44.44	41.67

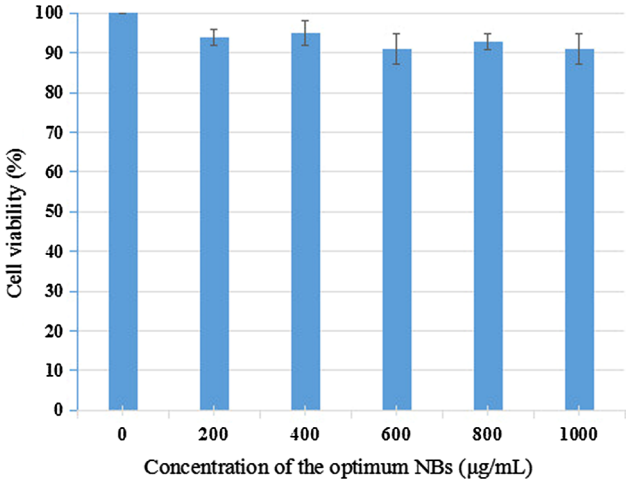

Fig. 8. *In vitro* cell cytotoxicity.

multifunctionality and possess the potential of controlled drug release profiles. The novel ST68/PLA-PEG stabilized NBs may be better suited for contrast enhanced imaging and subsequent therapeutic delivery in the future cancer-targeted applications.

Acknowledgement

This work was done in the Department of Ultrasound, Qilu Hospital of Shandong University.

This work was supported by the National Natural Science Foundation of China (NO. 81771843), the Science and Technology Developing Program of Shandong Provincial Government of China (2017GSF18107), the Key Research and Development Program of Shandong Province of China (2017GGX20141).

Declaration of Competing Interest

All the authors declare that they have no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultras.2019.105947.

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