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ORIGINAL ARTICLE

Phospholipid-based ultrasonic microbubbles for loading protein and ultrasound-triggered release

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Abstract

Background: Ultrasonic microbubbles are used as ultrasound-triggered delivery carriers for protein drugs. *Aim*: This work was to prepare stabilized protein-loaded phospholipid-based ultrasonic microbubbles (PUM) and to determine its value as a protein delivery system. *Method*: Bovine serum albumin (BSA) was used as a model protein drug. BSA-containing PUM were prepared by dissolving lyophilized PUM powder in BSA solution. The particle size and microbubble concentration of BSA-containing PUM were measured. The BSA encapsulation efficiency as a function of BSA concentration was determined. Contrast enhancement of BSA-containing PUM *in vivo* was detected. The release profile of BSA from PUM was also investigated. *Results*: The mean particle size and microbubble concentration of PUM were unchanged by the presence of BSA for at least 30 minutes after preparation. The net amount of BSA entrapped in PUM was maintained unchanged with increasing BSA concentration. BSA-containing PUM were shown easily to be visible in *in vivo* rabbit kidney. There was no difference in echogenicity between the loaded and unloaded PUM. Ultrasound duration had a positive relationship with BSA release. Ultrasound of 30 seconds stimulated 94.1% and 93.3% of BSA release from PUM solutions containing 0.3% and 1.5% BSA, respectively. *Conclusions*: Protein-loaded PUM exhibited satisfactory physical characteristics and were potent for using in ultrasound-triggered delivery.

Key words: *BSA; loading efficiency; microbubbles; ultrasound; ultrasound-triggered release*

Introduction

Ultrasound has been used in drug and gene delivery since the 1980s[1,2]. The drug delivery efficiency can be enhanced further if microbubbles are used in combination with ultrasound[3–5]. Peptide and protein or genes have strong physiological activity. However, most of these drugs cannot be used in their raw state, but have to be carried by suitable delivery systems, such as liposomes, micro- or nanoparticles, because of their instability and poor targeting ability in blood. Among these systems, the use of microbubbles as drug vehicles for control release has attracted considerable interest in recent years[6].

Many different compounds, such as acrylates, palmitic acid, phospholipids, albumin, and polymers, have been used as microbubble shells or surface-modifying agents. Among these, phospholipids are easily obtained chemically inert compounds that have no specific interactions leading to toxic reactions or side effects. Phospholipid-based ultrasonic microbubbles (PUM) are biocompatible and biodegradable echogenic agents of low toxicity. Many studies have highlighted their clinical application as contrast-enhancing agents. In our previous study, a self-made PUM agent has been proved as an effective ultrasound contrast agent[7]. Moreover, because of their bioadhesive properties, these PUM also have potential application in targeted gene/ drug delivery systems [8].

PUM delivery system enjoys many advantages in targeted drug delivery. Like other particulate systems, PUM can be loaded with therapeutic agents. Therapeutic gene/protein can be incorporated into the shell of PUM or attached to its surface. The PUM-carrying gene/protein can be tracked using ultrasound scanning.

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Active substances can be selectively released within designated organs by ultrasonic disruption of PUM as they transit or accumulate in the intended organ. Furthermore, the ultrasound-triggered delivery system may help to protect surrounding healthy tissue from significant levels of toxic drugs. The normal, nonultrasonic disruption of the microbubbles may eventually occur throughout the body, but with low levels of toxic material. Contrast-enhanced ultrasound images taken at various time points may prove to be useful in monitoring therapeutic progress and, thus, may verify efficacy claims for the marketing approval of a drug.

The cargo space available in the membrane of the microbubble is usually relatively small, which means that only potent protein drugs can be considered. Thicker shells can be used, but at the expense of ultrasound scattering efficacy [9].

One of the criteria for a successful PUM containing protein drug is that it must have the stability to survive ambient conditions during storage long enough for it to be used clinically [10]. The objectives of this work were to prepare stabilized protein-loaded PUM, to determine its value as a protein delivery system, and to demonstrate its in vivo imaging echogenicity. Bovine serum albumin (BSA) is a representative hydrophilic protein with a molecular weight of about 68 kDa. In this study, experiments were designed to prepare BSA-loaded PUM and investigate their physical stability, contrast enhancement in vivo, loading efficiency, and ultrasound-triggered release profile.

Materials and methods

Preparation of BSA-containing phospholipid-based ultrasonic microbubbles

The blank PUM was prepared by sonication–lyophilization method which was reported in our previous study[7,11,12]. The brief description of preparation was as follows. Hydrogenated phosphatidylcholine (HPC) (HPC > 99%, Doosan Corporation Biotech BU, Kyonggi Do, Korea), polyethylene glycol 1500 (Qingming Chemical Plant, Zhejiang Province, China), and poloxamer 188 (Shenyang Chemical Plant, Liaoning Province, China) were dissolved in normal butanol (analytical grade, Beijing Chemical Plant, Beijing, China) and sonicated at 30°C (JY 92-II ultrasonic processor, KunShan Ultrasound Instrument Inc., KunShan, China) at frequency of 40 kHz, power of 160 W for 3 minutes. The solution was stored at 0°C for 30 minutes and at –20°C for 1 hour. Then, the coagulated solution was lyophilized at 5×10^{-4} Pa pressure for 20 hours (primary drying at –48°C for 15 hours and then the temperature was gradually raised to 10°C within 5 hours). Lyophilized powder was put in 10 mL penicillin vials (200 mg/vial) and saturated with perfluoropropane (C_3F_8, electronic grade, Institute of Special Gas, Tianjing, China).

For the preparation of BSA (300,000 U/g, SINO-American Biotechnology Company, Shanghai, China)-containing PUM, 2 mL BSA solutions of different concentrations (0.3, 0.75, and 1.5 mg/mL) were added to 200 mg lyophilized powder, followed by gentle shaking to form an emulsion-like solution.

Characterization of BSA-containing phospholipid-based ultrasonic microbubbles

Morphologic characteristics of BSA-containing PUM were determined using an optical microscope (Olympus, Tokyo, Japan). Loaded and unloaded PUM suspensions were gently shaken before one drop was applied to the microscope slide. A cover slip was used to cover the sample before investigating the sample under a ×400 amplification. PUM loaded and unloaded BSA were viewed under the microscope at 0, 10, 30, and 60 minutes after addition of solvents, to observe the change of shape of microbubbles and to evaluate their stability. Particle size and concentration of microbubbles were analyzed on a Coulter counter (Coulter Corporation, Hialeah, FL, USA). For each sample, l0–15 μL of PUM solution was analyzed. The Coulter unit aperture was flushed with distilled water before and after use.

Loading efficiency of BSA-containing phospholipid-based ultrasonic microbubbles

BSA-containing PUM were considerably lighter than the surrounding aqueous phase. They could float to the top of the mixture by buoyancy force with long enough time. Therefore, free BSA could be separated from BSA-containing PUM and accumulated by centrifugation. Loading efficiency was calculated by measuring the concentration of free BSA. The detailed process is as follows.

Two milliliters of BSA-containing PUM suspension was centrifuged (110 g, 10 minutes) and the solution containing free BSA was separated. The supernatant microbubble layer was washed with 2 mL distilled water and the process was repeated. The underlayer solution containing free BSA was collected and BSA concentration was determined by Bradford's dye binding assay[13]. Coomassie Brilliant Blue G-250 (CBB G-250, Sigma, ShangHai Kenqiang Company, Shanghai, China) 100 mg was dissolved in 50 mL of 95% ethanol. To this solution 100 mL of 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 L. The final concentration in the reagent was 0.01% (w/v) CBB G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid. BSA standard solution was 0.1% (w/v). To BSA, deionized water was added based on various

proportions. Final concentrations in the protein solutions were 0.005%, 0.01%, 0.02%, 0.04%, 0.06%, 0.08%, and 0.1% (w/v) BSA, respectively. BSA solution of 1 mL and CBB G-250 solution of 5 mL, respectively, were added to the test tubes. The absorbance at 595 nm was measured after 5 minutes. Based on the result, the standard curve of Bradford's dye binding assay was described. The solution containing 0.1 mL of free BSA and 5 mL of CBB G-250 solution was added to the test tube. The content of free BSA was determined based on BSA standard curve. To eliminate background interference, blank PUM suspension was used as the control. Loading efficiency (%) of BSA-containing PUM was calculated using the following equation:

$$\text{Loading efficiency}\,(\%) = \frac{(\text{Total BSA} - \text{Free BSA})}{\text{Total BSA}} \times 100$$

Contrast enhancement in vivo

In our previous study, the blank PUM agent has been proved as an effective ultrasound contrast agent. However, ultrasound enhancement efficiency may be discounted with BSA adsorption in PUM. Therefore, it was necessary to revaluate the echogenicity of loaded PUM.

In contrast enhancement in vivo, contrast tuned imaging (CnTi) technique was used to assess the effects of BSA-containing PUM in normal rabbit liver parenchyma, because the area is easy to observe.

Four New Zealand white rabbits (male, 3.0 ± 0.2 kg, mean ± SD) were used in this study. The four rabbits were anesthetized with intramuscular injection of pentobarbital through a 20-gauge catheter in an ear vein. Bolus injections of the loaded and unloaded PUM solutions were also given through the catheter (the dose of the agent of each injection was 0.1 mL/kg). The tubing was flushed with 2 mL of saline after injection of PUM solutions. Each test condition was repeated four times (every rabbit was injected four times). Enhancement effect in rabbit liver parenchyma was recorded.

CnTi was done using a Technos^{MPX} DU8 (Parkson Group, Esaote, Italy). A convex probe, which has transmit/receive center frequencies of 2.63/5.26 MHz for second harmonic imaging, was used in this study. Acoustic pressure was 1 kPa. More than 15-minute interval was allowed between injections. Recording of ultrasound images was started just before injection and continued until the complete disappearance of the microbubbles.

Quantitative measurements using the images recorded on videotape were made to confirm the qualitative visual findings. The changes in video intensity of liver parenchyma were analyzed with an analysis system (Tomtec P90, Tomtec Imaging Systems Inc., Bolder, CO, USA). The image containing peak contrast enhancement was determined from the time–intensity curve, and the regions-of-interest (ROI) were set in the liver parenchyma. The average of the pixel intensities over the ROI was analyzed by computer (Macintosh PowerBook G3, Apple Computer Inc., Cupertino, CA, USA) using Adobe Photoshop^{TM} 3.0J (Adobe System Inc., San Jose, CA, USA), and the difference of maximum intensity between before and after enhancement was calculated. The duration of the contrast enhancement was also measured by time–intensity curves.

Ultrasound-stimulated release of BSA-containing phospholipid-based ultrasonic microbubbles

Ultrasound-stimulated release experiments were performed in a device as shown in Figure 1 immediately after lyophilized PUM samples were dissolved in BSA solution. PUM suspensions containing 0.3 mg/mL and 1.5 mg/mL BSA were added to the 12-well plates for ultrasound exposure. The ultrasound transducer (S3 transducer, Sonos 5500, Agilent Technology, Andover, MA, USA) was inserted in a 37°C water tank and directly faced the bottom of cell plate. A spongy rubber ultrasound shield was used to focus ultrasound on experimented cells. No ultrasound was detected outside of the rubber ultrasound shield. After BSA-containing PUM suspensions were added to the well, the 12-well plate was rotated manually at approximately 30 rpm for 20 seconds and exposed to ultrasound for two different durations (10 and 30 seconds). Each designed sample received ultrasound exposure in the water bath. The 12-well plate was held 4 cm from the submersed ultrasound transducer set to give a continuous 1.3 MHz ultrasound beam at a mechanical index of 1.2 for 30 seconds. After ultrasound exposure, PUM suspension in each well was transferred to centrifuge tubes. After centrifugation (110 g, 10 minutes), free BSA in solution was

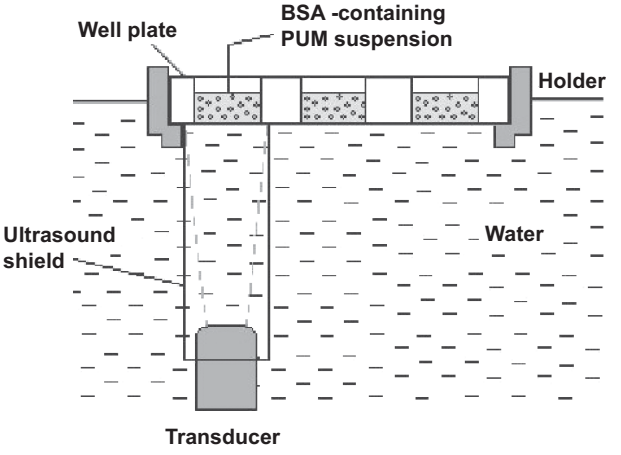

Figure 1. Ultrasound-triggered release device for BSA-containing PUM.

separated and measured by Bradford's dye binding assay mentioned as '2.3 Loading efficiency of BSA-containing PUM'. In each test, BSA-containing PUM suspension under same conditions without ultrasound treatment was used as the control. The amount of BSA release was calculated using the following equation. Release experiments of BSA-containing PUM without ultrasound treatment under same conditions were used as the contrast.

$$\text{BSA release (\%)} = \frac{(\text{Free BSA}_{\text{ultrasound}} - \text{Free BSA}_{\text{control}})}{(\text{Total BSA} - \text{Free BSA}_{\text{control}})} \times 100$$

Statistical analysis

Student's *t*-test and the one-way analysis of variation (ANOVA) were adopted for statistical comparison using the SAS 8.01 (1999–2000, SAS Institute Inc., Cary, NC, USA). The data difference was considered to be statistically significant when the *P*-value was less than 0.05.

Results

Morphology and size distribution of BSA-containing phospholipid-based ultrasonic microbubbles

BSA-containing PUM were prepared by dissolving lyophilized powder in BSA solution. The effects of the adsorption of BSA on PUM shell were shown in Table 1. BSA-containing PUM had the same mean particle size as that of unloaded PUM. No aggregation or fusion was observed in all PUM solutions in 60 minutes. The mean diameter and the morphologic characteristics, as seen under the microscope, of microbubbles did not change in different concentrations of BSA ($P > 0.05$).

Figure 2 shows the change of microbubble concentration of loaded and unloaded PUM over a period of 60 minutes. After contacting with hydrophilic solution containing different concentrations of BSA, lyophilized PUM were quickly converted into lipid-coated microbubbles and reached the highest microbubble concentration in 10 minutes. Then, the microbubble concentration gradually decreased over time. Compared with unloaded PUM solution, BSA-containing PUM solution showed little difference in microbubble concentratiowns at settling time ($P > 0.05$). Loaded and unloaded PUM solutions remained more than 2×10^8 microbubbles per milliliter in 60 minutes after dissolving in hydrophilic solution. The morphologic observation and size-distribution analysis showed that the existence of BSA had little effect on microbubble size and concentration.

Table 1. Change of microbubble mean diameter (μm) of loaded and unloaded PUM with time (mean ± SD, $n = 4$).

Solution	0 minutes	10 minutes	30 minutes	60 minutes
A	3.46 ± 0.13	3.45 ± 0.09	3.44 ± 0.10	3.45 ± 0.08
B	3.48 ± 0.14	3.47 ± 0.12	3.47 ± 0.11	3.47 ± 0.09
C	3.47 ± 0.12	3.48 ± 0.10	3.48 ± 0.09	3.48 ± 0.09
D	3.48 ± 0.13	3.48 ± 0.11	3.48 ± 0.12	3.48 ± 0.10

A, unloaded PUM solution; B, C, and D, PUM solutions loaded with 0.3, 0.75, and 1.5 mg/mL BSA, respectively.

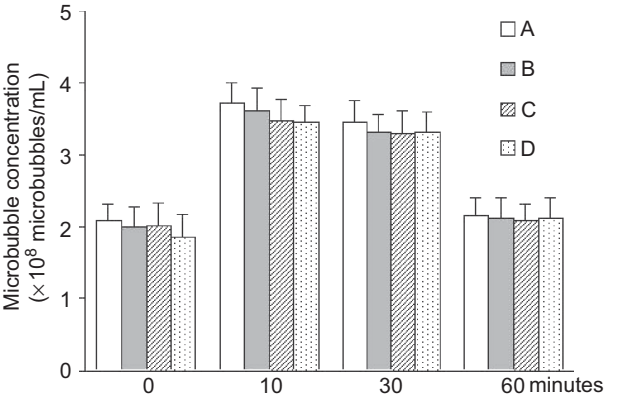

Figure 2. The average microbubble concentration of loaded and unloaded PUM solutions with time ($n = 4$). (A) Unloaded PUM solution; (B, C, and D) PUM solutions loaded with 0.3, 0.75, and 1.5 mg/mL BSA, respectively. The error bars stand for the SDs.

Loading efficiency of BSA-containing phospholipid-based ultrasonic microbubbles

As shown in Figure 3, the loading efficiency of BSA-containing PUM had an inverse relationship with BSA

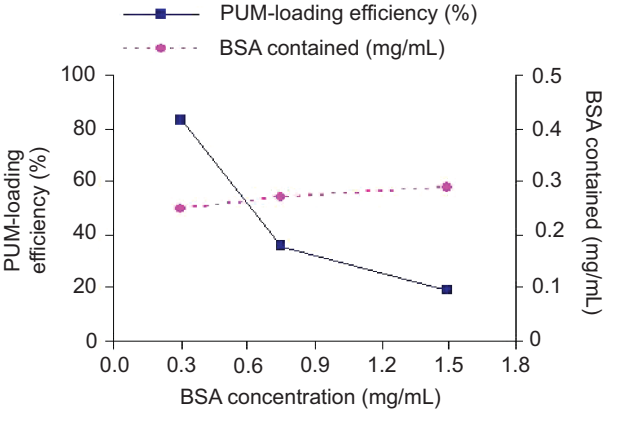

Figure 3. The average loading efficiency and net amount of BSA contained in loaded PUM with three different BSA concentrations ($n = 4$).

concentration. The loading efficiency of PUM declined quickly when BSA concentration was increased. However, the net amount of BSA contained per milliliter of PUM solution maintained relatively stable with increasing BSA concentration. These results demonstrated that the available loading volume within the membrane of PUM was limited.

Contrast enhancement in vivo

Contrast enhancement effect obtained from CnTi image in the liver parenchyma was shown in Figure 4. There was no significant difference ($P > 0.05$) in the contrast intensity and the longevity of CnTi enhancement between the images of the loaded and unloaded PUM (Table 2). Before enhancement, the relative intensity was less than 0.02 and had no significant difference among different test conditions. After intravenous bolus injection of PUM with or without BSA, over 200-fold (4.27–4.41/0.02) increase in contrast intensity was observed and the mean longevity of CnTi enhancement was more than 290 seconds. The results show that the ultrasound enhancement efficiency was not compromised with BSA adsorption in PUM. Therefore, loaded PUM could provide a targeted delivery system with imaging properties offering the monitoring of the targeted tissue.

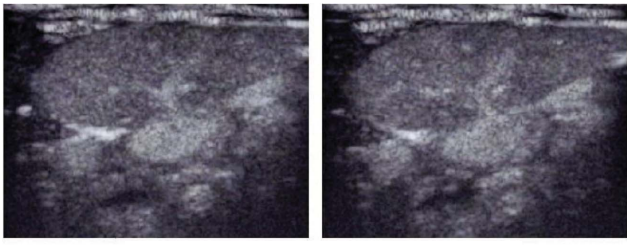

Figure 4. CnTi imaging of blank and loaded PUM in normal rabbit liver parenchyma: (a) blank PUM and (b) loaded PUM.

Table 2. Intensity and longevity of CnTi enhancement in the liver parenchyma (mean ± SD, $n = 4$).

Solution	Intensity (relative unit)	Longevity of the contrast effect (seconds)
Blank	0.02 ± 0.01	0
A	4.41 ± 0.14	301 ± 8
B	4.30 ± 0.16	298 ± 7
C	4.34 ± 0.15	302 ± 5
D	4.27 ± 0.13	295 ± 7

A, unloaded PUM solution; B, C, and D, PUM solutions loaded with 0.3, 0.75, and 1.5 mg/mL BSA, respectively.

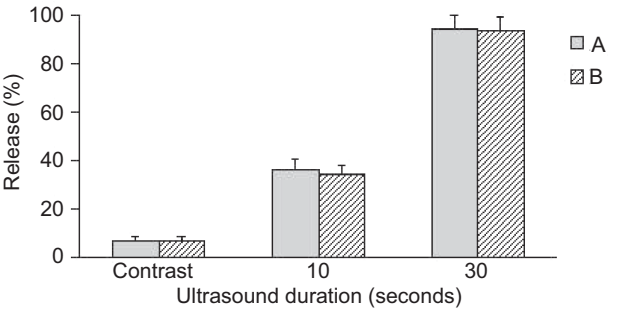

Figure 5. The average ultrasound-stimulated release from BSA-containing PUM ($n = 4$). (A) PUM solution loaded with 0.3-mg/mL BSA; (B) PUM solution loaded with 1.5-mg/mL BSA. The error bars stand for the SDs.

Ultrasound-stimulated release from BSA-containing phospholipid-based ultrasonic microbubbles

As shown in Figure 5, ultrasound duration had a positive relationship with BSA release. In contrast (without ultrasound treatment), only 7.2% and 6.8% of the entrapped BSA were released from PUM solutions containing 0.3% and 1.5% BSA, respectively. The applications of ultrasound of 10 seconds triggered 35.8% and 34.2% of the entrapped BSA release from PUM solutions containing 0.3% and 1.5% BSA, respectively. However, ultrasound of 30 seconds stimulated 94.1% and 93.3% of BSA release from PUM solutions containing 0.3% and 1.5% BSA, respectively. In addition, most of the microbubbles in solution disappeared after ultrasound of 30 seconds. As can be seen in Figure 4, PUM containing different concentrations of BSA showed little difference in BSA release percentage under same ultrasound exposure ($P > 0.05$). The unique release mechanism of ultrasonic microbubbles might contribute to explain the phenomenon. The release mechanism of loaded PUM is different from that of loaded microcapsules and loaded microspheres. Encapsulated drug was released from loaded PUM when microbubble shell was blasted under transient-enhanced ultrasound. Therefore, the release percentage of loaded PUM might have a close relationship with the action of devastating ultrasound wave.

Discussion

In this work, BSA-containing PUM were prepared and their physical stability, contrast enhancement in vivo, loading efficiency, and ultrasound-triggered release profile were investigated.

BSA is a hydrophilic protein with a molecular weight of about 68 kDa. From this study, showed that the

absorption of BSA in PUM had little effect on mean particle size and microbubble concentration. The total amount of BSA per milliliter of PUM solution maintained relatively stable. Because only the outer membranes of PUM are hydrophilic and can absorb water-soluble drugs, the cargo space available for protein loaded is limited. As the loading capacity of PUM was very small, only high-active biotechnology drugs were available in loaded PUM. In vivo imaging of the PUM has shown that they are easily visible. There is no difference in echogenicity between loaded and unloaded PUM. In this study, BSA-loaded PUM exhibited satisfactory physical characteristics. Most of the PUM maintained stable for at least 30 minutes. Ultrasound duration had a positive relationship with BSA release. Ultrasound of 30 seconds stimulated more than 90% BSA release from BSA-containing PUM. In clinical diagnosis, microbubble agent is required to be used as soon as possible after lyophilized agent dissolved in injection solvent. Ultrasound imaging diagnosis usually take 10–20 minutes. From the study, only decades of seconds are needed to finish the ultrasound-mediated release process in vivo. Therefore, the physical stability of PUM seems long enough to ensure ultrasound imaging and activate the release of loaded proteins.

Compared with traditional carriers, such as microspheres, liposomes, and microcapsules, the loading volume of PUM for protein drugs is small. However, traditional carriers are limited by the low efficiency of these vectors to cross biological barriers, including the blood vessels and cell membranes. Although the loading capacity of PUM is not superior than other microparticles, PUM enjoy many unique advantages in drug targeting delivery, such as offering ultrasound contrast image of targeted tissue, enhancing acoustic cavitations for transient permeability of plasma membrane[14], and inducing sonoporation effect to facilitate drug uptake[11].

As blood perfusion tracer, PUM can travel everywhere the red cells go, because the particle size and physical characteristics of PUM are similar to those of red cells. Many modification methods for PUM shell have been tried to improve PUM-loading capacity. In addition, it seemed that proteins with relatively low molecular weight have relatively high loading capacity. Unlike normal carriers depending on long time circulation to be accumulated in targeted area, ultrasound-mediated release mode needs only several minutes to finish the release process in vivo. Therefore, the duration of proteins exposed in blood circulation was greatly decreased. Denature and aggregate of proteins can be avoided because of shortened duration in blood circulation. In addition, using microbubbles as the ultrasound-mediated delivery carriers will require fewer amounts of the therapeutic proteins compared with normal delivery carriers. Therefore, the efficiency of PUM-mediated drug transfer may show much higher level than that of traditional carriers.

Although some people worried that ultrasound-mediated delivery system might alter the physical structure of the proteins and render the activity less stable, there were only few reports which supported the opinion. Most data reported in literature showed that ultrasound-mediated delivery system was able to increase the transfection efficiency and enhance expression of the genes and proteins[15–18]. With more methods used in improving the loading ability and active-targeting ability, PUM may be developed as 'magic bullets' to deliver therapeutic agents to the target region using ultrasound to activate release.

Conclusion

The application of PUM for drug delivery is currently in its infancy, especially for those highly active proteins. Various new PUM agents present a new era of ultrasound for future application in clinical medicine. Although we are far from the applicable system for clinical use, many reports show progresses in drug delivery applications in animal experiments.

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Declaration of interest: The authors report no conflicts of interest.

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