A Novel Fluoride Anion Modified Gelatin Nanogel System for Ultrasound-Triggered Drug Release

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ABSTRACT - Purpose. We prepared a novel fluoride anion-modified gelatin nanogel system and investigate its pattern of ultrasound-triggered drug release. **Methods.** Adriamycin (ADM) gelatin nanogel modified with fluoride anion (ADM-GNMF) was prepared by a modified co-precipitation method with fluoride anion and sodium sulfate. The loading and encapsulation efficiency of ADM were measured by HPLC. The size and shape of ADM-GNMF were determined by electron microscopy and photo-correlation spectroscopy. The diameter distribution changes and drug release efficiencies of ADM-GNMF, before and after sonication, were measured by a submicron particle size analyzer, an ultrasound generator, an ultrasound generator and an automatic sampler. **Results.** The ADM-GNMF was stable in solution with an average diameter of 46±12 nm; the encapsulation and loading efficiency of adriamycin were 87.2% and 6.38%, respectively. The ultrasound-triggered drug release and size change were most efficient at a frequency of 20 kHz, power density of $0.4w/cm^2$, and a 1~2 min duration. Under this ultrasound-triggered condition, 51.5% of drug in ADM-GNMF was released within 1~2 min, while the size of ADM-GNMF changed from 46 ± 12 nm to 1212 ± 35 nm within 1~2 min of sonication and restored to its previous size in 2~3 min after the ultrasound stopped. In contrast, 8.2% of drug in ADM-GNMF was released within 2~3 min without sonication, and only negligible size changes were found. **Conclusions.** The ADM-GNMF system efficiently released the encompassed drug in response to ultrasound, offering a novel and promising controlled drug release system for targeted therapy for cancer or other diseases.

INTRODUCTION

Recently, an increasing interest has been expressed in intelligent drug delivery systems that have the capacity for triggered release by external factors, such as temperature, pH value, ultrasound, or electromagnetic fields. These systems' phase volumes can be changed greatly in direct response to environmental factors and restored to their original phase volumes immediately after the environmental factors are removed (1-3). Among these environmental factors, ultrasound is an attractive approach to controlling drug release, as it is non-invasive yet able to penetrate deep into the interior of the body. Furthermore, ultrasound can be focused on targeted sites and has been shown to increase the permeability of blood-tissue barriers and cell membranes (4). Several systems of ultrasound-triggered drug release, specifically the poly (DL lactide-co-Glycolide)(PLG) system (5) and acoustically active liposomes (6), have been reported. However, not all of the ultrasound triggered release systems can be used clinically because of their low sensibility in response to

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ultrasound stimulation and poor drug loading. For example, over 10 min of sonication time is required to trigger 20% of drug release from the PLG system (5). More recently, the pluronic P105 micelle system has attracted much interest. The system has a special structure formed with amphiphilic molecules in an aqueous medium. Its hydrophilic poly(ethylene oxide) (PEO) portion presents itself to the external aqueous media, while the hydrophobic part, poly(propylene) (PPO), is oriented towards the internal part of the structure. The system is sensitive to reversible ultrasound-triggered release (only a 0.5~1 min duration is needed). The pluronic system has been studied by Rapoport et al. (7, 8, 9); this group has investigated this system's factors affecting drug delivery, the mechanism of the ultrasound-triggered release, and targeting therapy to tumors *in vitro* and *in vivo*. However, there are still challenges to overcome, since pluronic P105 formulations are associated with either lower quantity of drug uptake and/or release (e.g., <12%) by ultrasonic trigger compared to conventional drug carriers. This is attributed to the low critical micelle concentration (CMC) of pluronic micelles and the smaller (10~50nm) micelle size. On the other hand, the molecular mechanism of drug-cell interaction and ultrasound-triggered effects on this process have not been revealed thoroughly; this includes the interaction of the ultrasound with specific molecules in the outer region or the core of the pluronic P105 micelle or with the drug, as well as cell effects (8). Alexander et al. realize that transient cavitation plays an important role in triggering drug release from pluronic P105 micelles; shock waves produced by transient cavitation events disrupt micelles and release drug to the environment (8). This finding could not be accepted generally without more insight into the mechanism involved.. Therefore, developing more sensitive ultrasound-triggered release systems with higher drug loading capacities, as well as studying their molecular mechanisms are timely.. Natural compounds are optimal to serve as

drug delivery carriers. It is known that gelatin is a natural polymer consisting of various peptides, and has the characteristics of good biocompatibility, non-toxic degradation, and ability to readily release drugs.. Furthermore, various forms of gelatin carriers can be fabricated for controlled release studies (10). The main drawback of rapid solubilization in aqueous environments can be overcome by chemical cross-linking with glutaraldehyde, formaldehyde, etc. In a previous study, we have prepared gelatin microspheres (GMs) containing pingyangmycin hydrochloride using oxidized dextran (ox-dex) and glutaraldehyde as the cross-linking agents. These microspheres can increase the local drug concentration and targeting therapy (11). Our previous studies proved that the puckered peptides of protein can be stretched under suitable ultrasound conditions (12, 13, 14). Since fluoride anions have the powerful ability to attract electrons and can easily establish hydrogen bonds with oxygen and nitrogen atoms in polymers, we hypothesize that the incompact structure of gelatin nanogel can be established when it contain a certain quantity of fluoride anions. The nanogel has adjustable small clearances or holes, and can be easily extended and shrunk partly due to the extension of peptides when exposed to ultrasound. To design a sensitive and higher drug-loading ultrasound-triggered drug release system with gelatin, it is important to produce transient cavitation inside its network. In the present study, we have developed a novel ultrasound-triggered drug release system made of gelatin nanogel modified with fluoride anion and characterized its ultrasound-triggered drug release. The results demonstrate that our gelatin nanogel modified with fluoride anion is a novel and promising ultrasound-triggered drug release system.

MATERIALS AND METHODS

MATERIALS

Gelatin G-6650, Tween-20, and Span-20 were obtained from Sigma Inc (Sigma Chemicals, Saint-Louis, MO, USA). Sodium fluoride and Dextran T40 were purchased from Xiasi Biotechnology Co. (Beijing, China). Ox-dextran was produced with Dextran T40 in our laboratory (11). Standard adriamycin was supplied by the National institute for the control of pharmaceutical and biological products (Beijing, China). Adriamycin was purchased from Taihe Pharmaceutical Inc. (Tianjin, China). 6KD dialyzer was purchased from Sino-American Biotechnology Company. All chemicals and solvents were used without further purification. Water was double-distilled.

Preparation of adriamycin gelatin nanogel modified with fluoride anion (ADM-GNMF)

The preparation method involved co-precipitation with fluoride anion and sodium sulfate. Briefly, 10%w/w cross-linked gelatin solution was obtained as follows: 5 ml of water containing 112 mg of ox-dextran was reacted for 10 min at 60ºC with 1g of gelatin dissolved in 3 ml of water (11). Then, 10 mg of adriamycin was introduced to 10ml 10% w/w cross-linked gelatin solution containing 1.8% (v/v) Tween-20 and 0.8% (v/v) Span 80 ; the pH value of the gelatin solution was adjusted to 4.5, then heated to 50 °C and stirred for 5 min. Afterwards, 4.5 ml of 0.50 mol/l sodium fluoride was added dropwise to this solution, which was maintained at a final concentration of 3% w/w fluoride anion in solution. 6.0 ml 20% w/w of sodium sulfate solution and 6.0 ml isopropanol were added dropwise to this system, which was maintained at 0-5 °C with an ice water bath. The mixture solution became muddy, and then became clear. This system was sonicated using an ultrasound generator at 75w with a frequency of 20

kHz (CP 101, Cole-Parmer International Inc, U.S.A) for 1 min, and then repeated three times, and the pH value of the system was adjusted to 7.8. The resulting adriamycin gelatin nanogel modified with fluoride anion (ADM-GNMF) was washed three times with water and passed through a Sephadax G-75 column to separate the free adriamycin from the ADM-GNMF solution. About 76% of the yield in this process is obtained. The final concentration of adriamycin was adjusted to 0.50 mg/ml. The resulting purified ADM-GNMF was freeze-dried to a powder, and stored at 4°C.

Morphology and size analysis

The morphology of ADM-GNMF was evaluated by transmission electron microscopy (TEM, HITACHI S-520, Tokyo, Japan) observations. The size distribution was characterized by particle size distribution analysis using a Beckman Coulter N4 plus submicron particle size analyzer utilizing a photo-correlation spectroscope (15). The morphology photographs of ADM-GNMF in different times with or without sonication were recorded by a digital camera (DSC-S600, Sony, Japan).

Determination of drug encapsulation efficiency and loading

Exactly weighed amounts of ADM-GNMF (100 mg) and GNMF nanogel (100 mg) were treated with 50 mL NaOH (10%, w/v) solution for 20 min at 80°C. The fully degraded gelatin solution was adjusted to neutral pH with 3 mol/l HCl, and then centrifuged for 5 min. The supernatant was mixed with total-ionic-strength adjusting buffer (TISAB) and introduced into a 50 mL solution. The drug loading and encapsulation efficiency were determined by means of a high performance liquid chromatography (HPLC) (Waters 510, Water Inc, U.S.A) device with a fluorescent measurement at an excitation wavelength of 500 nm and an emission

wavelength of 550 nm. The mobile phase was a mixture of methanol, 0.0l mol/l ammonium dihydrogen phosphate, and acetonitrile with the volume ratio of 7:30:0.5. The chromatography was performed by injecting 50 μl of sample into the column, followed by running the mobile phase at a flow rate of 1.0 ml/min (18). The standard curve peak areas on the chromatograms as a function of the ADM concentration were also studied by HPLC. The actual amount of ADM found can be determined by using a standard curve. The drug loading and encapsulation efficiency were calculated as below:

Encapsulation efficiency (%)

$$= \frac{\text{Weight of ADM found}}{\text{Weight of ADM input}} \times 100\% \qquad (1)$$

Loading efficiency (%)

$$= \frac{\text{Weight of ADM found}}{\text{Weight of ADM - GNMF}} \times 100\% \qquad (2)$$

Stability of ADM- GNMF

Dry ADM-GNMF was sealed and deposited in a 4°C refrigerator and at room temperature for six months, respectively. The appearance, morphology, and drug content of ADM-GNMF were examined according to the methods described above.

In Vitro **drug release of ADM-GNMF**

In vitro drug release profiles were assessed by a dynamic dialysis method. The release experiments were conducted at 37ºC. Typically, 50 mg of ADM-GNMF were placed into a dialysis bag and placed in 100 ml of 0.1 mol/l phosphate-buffered solution (PBS, pH 7.4) with magnetic stirring at 200 rpm. At certain time intervals, 5ml of sample were removed from the release medium, and the same volume of PBS was added to this system. Then, the samples were assayed for drug content

according to a standard curve using HPLC. According to Pharmacopoeia of China (19), a series of standardized ADM solutions: 64, 32, 16, 8, 4, and 2μg/ml was prepared and 20μl were injected for experiments; using ADM content C (μg/mL) and peak area V (m AU·s) as parameters, a linear regression equation was obtained by HPLC: C=2.7566V+0.0343, correlation coefficient: r=0.9987, linearity range: 2-64μg, relative standard deviation: RSD<2%. The same experiments were performed again after ADM-GNMF was deposited in a 4ºC refrigerator and room temperature for 6 months.

Characterization of ultrasound-triggered drug release

Two real-time ultrasound-triggered release measuring systems are schematically shown in Figure 1. The first, shown in Figure 1(a), consisted of a Beckman Coulter N4 plus submicron particle size analyzer and an ultrasonic transducer was inserted into the sample pool; the temperature of the sample pool was maintained at 37ºC by a circulating water bath throughout the experiments. This system can measure the average size and size distribution of samples in real-time with different ultrasonic parameters. Another real-time ultrasound-triggered release measuring system is shown in Figure 1 (b); an ultrasonic transducer was inserted into the sample pool separated by a semi-permeable membrane at 37ºC. When ultrasound was switched on, the small molecule drug was released and penetrated the other side of the semi-permeable membrane rapidly when the drug concentration in whole pool was kept constant. Sample were removed from the pool using Automatic samplers (GPRT2-70, Index Inc) within 1~3s. The real-time drug concentrations taken from the pool sample with ultrasonic triggering can be obtained through HPLC with fluorescence intensity measurements at various time points.

The characterization of ultrasound-triggered drug release was performed as follows: an exactly weighed amount of ADM-GNMF was dispersed in PBS, pH7.4, and placed in sample pool as in Figure 1 (a). Ultrasound is generated by a transducer, which can sonicate 1~100 kHz, 0.2~20 w/cm^2 ultrasound. The real-time average size and size distribution of ADM-GNMF were recorded by Beckman Coulter N4 plus submicron particle size analyzer. In addition, the amount of drug release from the sample pool in Figure 1 (b), before and after sonication, was measured using the HPLC method described above. 40μl of sample were removed from the release medium with the automatic sampler, and the same volume of PBS was added back to the release medium. As the ADM concentration at different times was obtained, the drug release was calculated as follows:

Drug release %

$$= \frac{\text{weight of free ADM}}{\text{weight of ADM - GNMF}} \times 100\% \quad (3)$$

Where weight of free ADM can be determined by the HPLC method, weight of ADM in ADM-GNMF can be calculated from the drug loading and encapsulation efficiency of ADM-GNMF. Temperature was maintained at 37°C in all the experiments. After preliminary exploration of conditions, 0-90 kHz, 0-2 W/cm^2, and 0-140 μg/ml drug were chosen for subsequent experiments. As a control, the same amount of ADM-GNMF was dispersed in PBS, and the average size, size distribution, and the amount of drug release were determined without sonication. The same experiments were repeated after ADM-GNMF was stored at 4°C for 6 mouths.

STATISTICAL ANALYSIS

Data are expressed as means ± S.D. calculated from at least three independent experiments with the SPSS 11.0 software (SPSS Inc., Chicago, USA); one-way ANOVA followed a post hoc Dunnett test was applied to the data, and a p-value less than 0.05 was considered as a statistically significant difference between two compared data.

RESULTS

Morphology and particle size

The TEM photograph of the ADM-GNMF shows a smooth and uniform surface, which was stable with good fluidity when stored at 4°C or at room temperature. The average diameter of ADM-GNMF is 46 ± 12 nm (Fig2). After storage at 4°C for 6 months, only negligible differences in ADM-GNMF morphology and particle size were observed (Figure 3).

Encapsulation efficiency and drug-loading

As describe above, the ADM concentration can be measured by the HPLC method, and the drug-loading and encapsulation efficiency can then be calculated according to the Equations 1-3. The encapsulation and loading efficiency of adriamycin were 87.2% and 6.38%, respectively. Only negligible difference in drug encapsulated efficiency and loading can be found after storage at 4°C for 6 months (Figure 3).

In Vitro drug release of ADM-GNMF

The characteristics of drug release were tested *in vitro* by the dynamic dialysis method with HPLC. The release profiles of ADM from ADM-GNMF are shown in Figure 3. It was found that about 48.5 ± 3.1% of the total amount of the encapsulated drug was released after 50 h at 37ºC..
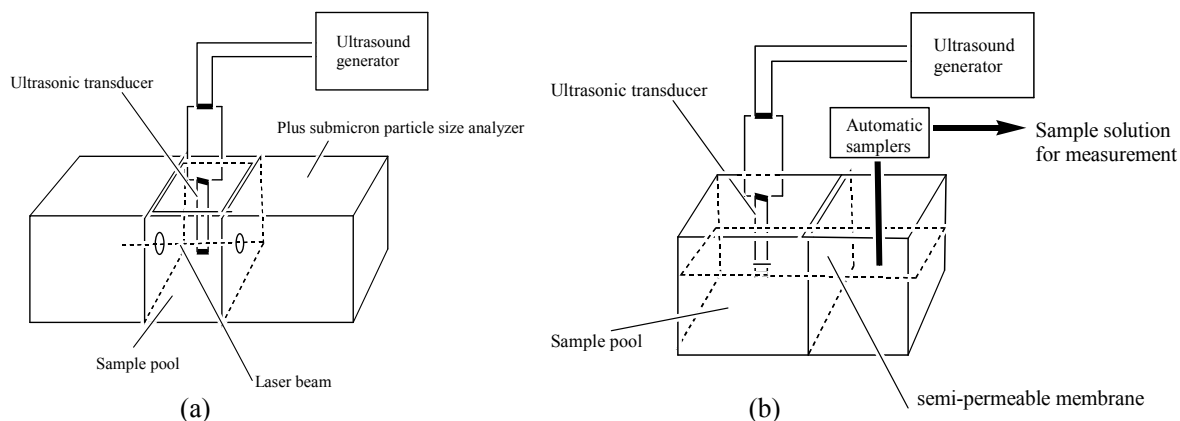
Figure 1. Ultrasound-triggered drug release measuring systems (a) the real-time average size and size distribution measuring system consists of submicron particle size analyzer and ultrasound generator: (b) the real-time drug concentration measuring system consists of the sample pool, automatic samplers, and ultrasound generator.
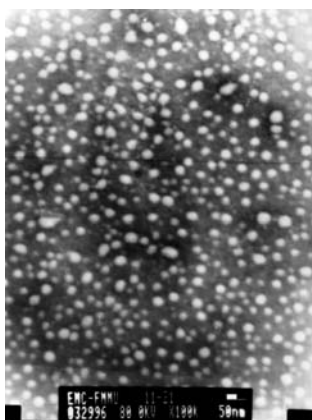

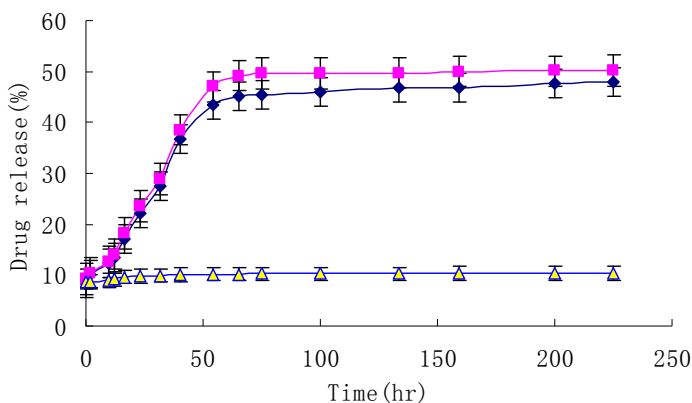**Figure 2.** Transmission electron microscopy photograph of ADM-GNMF.

Figure 3. *In vitro* drug release profiles of ADM- GMNF（□ADM-GMNF at 37ºC , ■ ADM-GNMF at 37ºC after storage at room temperature for 6 months, □ADM-GNMF after storage at 4ºC after storage for 6 months, n=4).
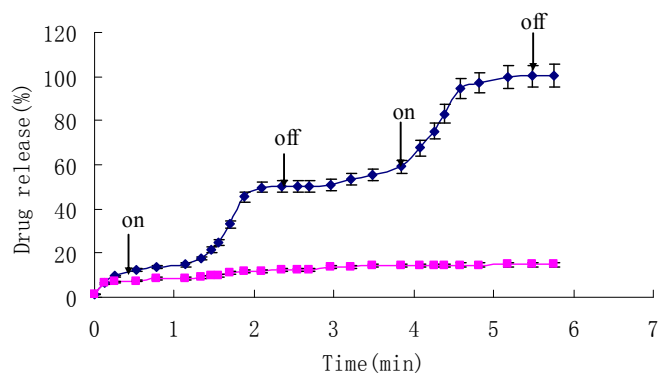
Figure 4. ADM release profile from ADM- GNMF upon sonication；ADM concentration was 20μg/ml, ultrasonic frequency was 20 kHz, power density was 0.4w/cm^2. （■ ADM-GMNG in PBS as a control group, ◆ADM-GNMF at sonication , n=4).
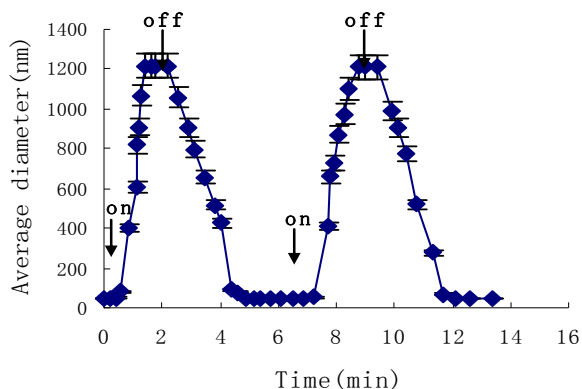

Figure 5. Average diameter change profiles of ADM-GNMF upon sonication. ADM concentration was 20μg/ml; ultrasonic frequency was 20 kHz, power density was 0.4w/cm^2 （n=4).
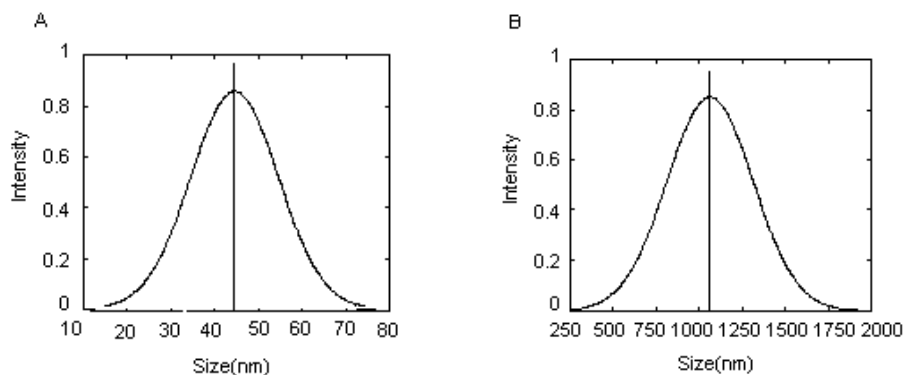

Figure 6. Diameter distribution changes of ADM-GNMF before and after sonication. ADM concentration was 20μg/ml; ultrasound frequency was 20 kHz; power density was 0.4w/cm^2; 7.5 min.
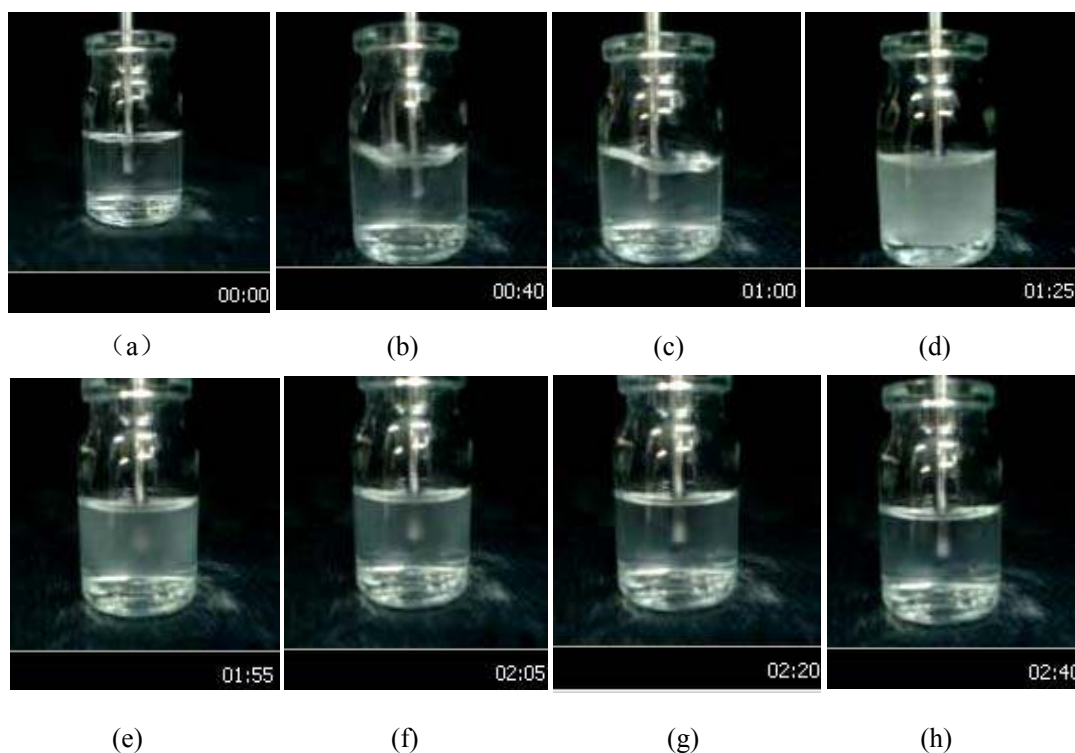
Figure 7. Morphology photographs of ADM-GNMF at difference time points. (a)-(d) sonication process; (e)-(h) restored process; ultrasonic frequency was 20 kHz; power density was 0.4 w/cm^2; volume was 5.0 ml.
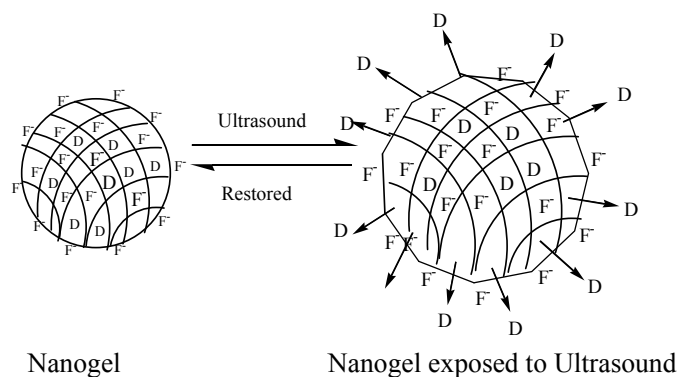

Figure 8. A possible mechanism of ultrasonic triggered release (D is drug and F$^-$ is fluoride anion).

In contrast, a slight increase in drug release (about 50%) was found after ADM-GNMF was stored at room temperature for 6 months and no statistical difference between the two samples can be found. Only about $10 \pm 1.3\%$ of encapsulated drug was released at 4ºC after ADM-GNMF had been stored at 4ºC for 6 months. Our results indicate that the encapsulated drug can be released from ADM-GNMF, which was stable with good fluidity, the appearance and loading effeciency of ADM-GNMF hardly occur any change when sealed and stored under common conditions, such as in the

refrigerator or at room temperature for six months.

Characterization of ultrasound-triggered drug release

The characterization of ultrasound-triggered drug release of ADM-GNMF was performed with the measuring systems shown in Figure 1. Examples of the release profiles of ADM from ADM-GNMF are shown in Figures 4 ; the enhanced drug release during the ultrasound "on" indicates the rapid ultrasound- triggered drug release effect. As shown, the diameter of ADM-GNMF changed from 46±12 nm to 1100±35 nm after ultrasound had triggered for 1~2 minutes, meanwhile the ADM content of ADM-GNMF that was released increased from 8.2% to about 50%. Figure.5 reveals the average diameter change profiles of ADM-GNMF under the action of ultrasound. A fast return to original size is

observed in ADM-GNMF after the ultrasound is turned "off" for 3 mins (Figure 5). It is noteworthy that the phenomena of the drug re-encapsulation wasn't found, which was different from the the phenomena of pluornic P105 micelles system (7, 8, 20). The size distribution of ADM-GNMF before and after sonication confirms this result (Figure 6). The Morphology and particle size changes of ADM-GNMF at different time points after sonication are shown in Figure 7. It is shown that significant morphology and particle size changes of ADM-GNMF can be observed after sonicating for 1~2 minutes, while morphology and particle size can be restored after sonication is stopped for 2~3 minutes. These results are consistent with the average diameter change profiles of ADM-GNMF upon sonication in Figure 5.

Table 1. Effect of different frequencies on the characteristics of ultrasound-triggered drug release

Frequency（kHz）	0	20	30	40	60	80	90
Drug release（%）	8.3±0.5	50.9±3.2[a]	50.6±3.6[a]	48.7±3.0[a]	37.3±2.8[a]	37.4±2.5[a]	36.6±2.4[a]
Diameter （nm）	46±12	1052±35[a]	1029±32[a]	798±25[a]	565.5±22[a]	564.7±19[a]	562.5±21[a]

Note: 3 min; drug concentration was 100 µg/ml; power density was 0.4 W/cm^2; n=4. [a]$P<0.01$ compared with 0 kHz frequency.

Table 2. Effect of different power density on the characteristics of ultrasound-triggered drug release

Power density （w/cm^2）	0	0.2	0.4	0.6	1.0	1.2	1.4
Drug release（%）	8.2±0.6	32.6±1.2[a]	50.2±3.2[a]	42.2±3.6[a]	38.1±3.3[a]	32.6±2.8[a]	31.8±3.0[a]
Diameter （nm）	46±12	1126±28[a]	1082±30[a]	1036±24[a]	1036±26[a]	1047±22[a]	1038±25[a]

Note: 3 min; ultrasonic frequency was 20 kHz ; drug concentration was 100µg/ml ;n=4. [a]$P<0.01$ compared with 0 w/cm^2 power density

Table 3. Effect of different drug concentration on the characteristics of ultrasound-triggered drug release

Drug concentration （µg/ml）	0	20	40	60	100	120	140
Drug release（%）	0	51.5±4.0[a]	50.4±3.6[a]	48.6±3.9[a]	46.3±4.4[a]	45.6±3.1[a]	44.8±3.2[a]
Diameter （nm）	47±11	1212±35[a]	1110±25[a]	1080±23[a]	1032±22[a]	1030±25[a]	1026±21[a]

Note: 3 min; ultrasonic frequency was 20 kHz; power density was 0. 4W/cm^2; n=4. [a]$P<0.01$ compared with 0 µg/ml drug concentration.

Table 4. Effect of different pH values on the characteristics of ultrasound-triggered drug release

pH values	5	6	7	8	9	10
Drug release（%）	48.3±3.2	50.2±3.9	51.5±4.2	49.1±3.0	46.3±4.4	25.6±2.2[a]
Diameter （nm）	1002±12	1032±36	1211±37[b]	1080±23[a]	962±32	972±29

Note: 3 min; ultrasonic frequency was 20 kHz; power density was 0. 4W/cm^2; drug concentration was 20μg/m, n=4. [a]$P<0.05$, [b]$P<0.01$compared with pH=5 value.

Our results show this system has the characteristics of reversible and continuous ultrasound-triggered drug release effects. More importantly, we explored ADM release as the function of ultrasonic frequency within the frequency of 0~90 kHz at a lower power density of 0.4 W/cm^2 (Table 1). ADM release from ADM-GNMF was most efficient at 20 kHz, while the diameter of GNMF had the maximum value after ultrasound was triggered for 1~2 min. This result is consistent with the observation of the effect of ultrasonic frequency on the pluronic P105 micelles (7). As shown in Table 2, the maximum value of ADM release was achieved in the 0.4 w/cm^2 power densities at the same frequencies and ultrasonic times as in Table 2. While other influence factors have the same values as Table 2, the effect of ADM concentration on the ultrasound-triggered drug release characteristics was also examined. As shown in Table 3, there are no obvious differences between ADM release from 20 to 140 μg/ml ($P>0.05$); the ADM release rate of ADM-GNMF increases as the ADM concentration decreases. Meanwhile, there are significant size differences when the ultrasound is "on" or "off". For example, at a concentration of 20 μg/ml ADM, the drug release rate and nanogel size were 44.8% and 1212 ± 35 nm after sonication for 7 min, while at a concentration of 140 μg/ml of ADM, the drug release was only 51.5% and the size remained at 1026± 21 nm ($P<0.05$). The higher drug release rate at the lower drug concentration could be attributed to a steady peak release (Figure 4), i.e. whatever the changes of the drug content in the ADM-GNMF system, the peak release rate within

certain time has the same value (about 50%); therefore, a lower drug concentration will gain a higher drug release rate. According to the results described above, the optimal release conditions of ADM-GNMF were as follows: frequency of 20 kHz, power density of 0.4 w/cm^2, a 1~2 minute duration, and 20 μg/ml of ADM. Under these conditions, 51.5% of adriamycin in ADM-GNMF was released, and the size of ADM-GNMF changed from 46 ± 12 nm to 1212 ± 35 nm; this was significantly higher than the control group ($P<0.001$). In addition, we are still investigating the effects of other conditions, such as temperature, pH value, and ionic strength on the characteristics of ultrasound-triggered drug release. Ionic strength had the highest effect on the release characteristics of ADM-GNMF. For example, the ionic strength of this system should be maintained at 0.50 mol/l, which is mainly adjusted by the concentration of the fluoride anion. Even a 0.2 mol/l difference in ionic strength in the ADM-GNMF system can change its release characteristics. When the ionic strength of the ADM-GNMF system was adjusted to the outward region of the 0.50 ± 0.2 mol/l range, the effect of ultrasound-triggered drug release disappeared immediately.This result proves that the ultrasound-triggered drug release effect only appears in certain ranges of fluoride anion concentrations. The results could be attributed to the nanogel structure in the hydrophilic core, in which ADM is dissolved in the solution at pH 5.0~8.0(Table 4). Therefore, the characteristics of ultrasound-triggered drug release cannot be found when the pH value of the system is over 10 or less

5.0. On the other hand, there are negligible differences in drug release when the exterior temperature is changed from 0~45° C. In contrast, ADM-GNMF will be precipitated into larger sized particles and the solution can become solid when stored lower than 0°C, which can counteract transmission of ultrasound waves. The network structure of ADM-GNMF can be destroyed when temperature is increased over 45°C. The temperature should be maintained within a certain range which will not interfere severely with the drug release of ADM-GNMF. Finally, our experiments also show that ultrasound-triggered drug release of ADM-GNMF did not change after storage at 4°C for over one year.

DISCUSSION

Recently, several different ultrasound-triggered drug delivery systems have been reported. Rapoport N et al reported that ultrasound at frequencies of 20 to 70 KHz and a power of 2 or 1 w/cm^2 gave rise to release of up to 12% of drug contained within pluronic micelles (7, 8). As described in another system, Unger et al entrapped a hydrophobic drug into lipid shells of gas-filled microspheres by incorporating soybean oil in the shell. Sixty percent of the microspheres were released by application of 2.5 MHz ultrasound at 0.8 w/cm^2 for 30 min (15). More recently, Huang S.L et al reported that approx 30% of drug was released from acoustically active liposomes after sonication at 1 MHz and 2 w/cm^2 (6). In our experiments shown here, 51.5% of drug in ADM-GNMF was released within 2-3 min after sonication at 20 KHz and 0.4 w/cm^2. Meanwhile, the particle size of this system is increased greatly and restored rapidly, which is different from the ultrasound-triggered drug delivery systems reported by others. Our system appears to be one of the most sensitive and useful ultrasound-controlled release systems described so far. Additionally, the real-time measurements of average size, size distribution, and

ADM concentration of ADM-GNMF upon sonication were the most critical experiments in this study. G.A Husseini et al designed a real-time measurement system of ultrasound-triggered release using a special ultrasound exposure chamber with fluorescence detection; the micellar percentage of drug release was calculated by the formula: Release (%)=[(F$_{mic}$-F$_{us}$)]/(F$_{mic}$-F$_{pbs}$)] %, where F$_{pbs}$ is the fluorescence intensity of the drug in phosphate buffer, F$_u$ is the fluorescence intensity of pluronic micelles, and F$_{mic}$ is the fluorescence intensity of the mixture solution(including micelles and released drug) (17). Huang SL et al designed a similar improved real-time fluorescence intensity measurement device which can detect small amounts of drug concentrations during sonication of acoustically active liposomes (6). Those methods have special devices which can be easily interfered with by the existing micelles or liposomes in solution, and more importantly, those methods cannot detect real-time size and size distribution of the system exposed to ultrasound. In our experiments, the existence of ADM-GNMF nanogel in solution will interfere with the detection of released drug during exposure to ultrasound. In addition, the volume of the system changes greatly before and after sonication, the drug concentration in the whole pool was stable within 10~15 s due to the penetrating character of the ultrasound, the real-time drug concentration can be detected in the pool within 1~3s, and the difference in compositions can be separated by an HPLC method and detected with fluorescence thus avoiding the interferences of the nanogel, its volume changes, as well as other compositions to the detection results.

Gelatin is a common natural polymer consists of peptides, which can be fabricated into various forms of controlled-release drug delivery systems. More importantly, drugs or bio-molecules released from gelatin controlled-release drug delivery systems are able to retain their biological activity, allowing for their use in clinical application (16). Our current system provided not only a novel

gelatin controlled-release drug delivery system but also a novel strategy for targeting therapy. The mechanism of improved drug release by ultrasound has been studied for several years. In spite of several published studies, the mechanism is still not well understood or characterized. A possible mechanism of improved drug release from pluronic P105 micellessuggested by Rapoport N, et al. is that the ultrasound shifts the equilibrium between the free drug and micellar-encapsulated drug toward the free drug and affects the series of equilibriums in cells (7, 8). Another possible mechanism is that ultrasound might interact with the structure of the micelle located in the channels of the drug release (9, 17). All recent studies indicate that cavitation plays an important role in enhancing the drug release mechanism; therefore, cavitation may play a significant role in our ADM-GNMF system. In this study, as Fig.7 shows, a lot of cavitation and an increasing volume of ADM-GNMF appeared at the same time, and restoration time was longer than triggering time. Figure 4 show the phenomena that released drug didn't re-encapsulate to nanogel when ultrasound stopped; this process can repeat many times until the encapsulated drug is released entirely. The maximum drug release time is consistent with the largest volume of the nanogel shown in Figure 5. As described above, a possible mechanism of ultrasound-triggered drug release is shown in Figure 8. Nanogel has the network structure integrity, in which the high solvent content gives rise to the fluid-like transport properties, and drug and large quantity of fluoride anion are contained inside and on its surface. Compared to other ions,such sodium, potassium, calcium as well as anion of chlorine, bromine and iodine, fluoride anion has a powerful ability to attract electrons and can easily establish hydrogen bonds with the oxygen of water, this is probably the reason that the ionic strength had the highest effect on the release characteristics of ADM-GNMF. If the ionic strength of fluoride anion was higher enough, the interaction between fluoride anion to protein molecule of the gelatin was powerful and combined each other, thus larger gel would be formed. In contrast, the ionic strength of fluoride anion was so lower that the interaction between fluoride anion to protein molecule of the gelatin was weak, the gelatin nanogel couldn't be existed. Therefore, the suitable ionic strength of fluoride anion is 0.50 ± 0.2 mol/l.

Our previous studies prove that the puckered peptides of protein can stretch under ultrasound conditions (12,13,14). Therefore, while nanogel is exposed to ultrasound waves, they permeate the surface layer and provide energy to the system that can interrupt the hydrogen bond and network structure of nanogel. In addition, the peptides of gelatin are stretched under ultrasound. Consequently, the phase volume of the nanogel increased greatly and the drug could not be re-encapsulated. With the effects on cavitation continuously produced, disappearing and disrupting nanogel, ADM dissolved in water comes out of nanogel with cavitation, the combined effects above then cause the enhancing of the drug release. Another aspect is due to the fact that the ADM-GNMF is the core of the cavitation at sonication, and a large amount of cavitation is congregated on its surface. The process of cavitation's production, movement, disappearing, and shock enhances the holes on the surface, thereby increasing the quantity of drug release significantly. In contrast, the large quantity of cavitation disappears when ultrasound stops, and the system has the tendency to regain a stable structure. Thus, the nanogel is restored to its previous size after a longer time. The proposed mechanism of ultrasound-triggered release described above is just our hypothesis; the exact underlying mechanism or molecule interaction of ultrasound- triggered drug release is complex and remains to be delineated.

In conclusion, in the present study, we have established a novel and promising

ultrasound-triggered drug delivery system, ADM-GNMF, with sensitive and robust ultrasound-triggered drug release properties, which can be developed into an effective therapeutic technology platform for targeted and controlled drug release delivery system for cancer and other diseases.

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REFERENCES

[1]. Anderson, D.G., Burdick, J.A., and Langer, R.S. Smart Biomaterials. Science, 305: 1923-1924, 2004.

[2]. Kost, J., and Langer, R.S. Responsive polymer delivery systems. Adv Drug Deliv Rev, 46: 125-148, 2001.

[3]. Gil, E S., and Hudson, M.S. Stimuli-responsive polymers and their bioconjugates. Prog. Polym Sci, 29: 1173-1222. 2004.

[4]. Uhrich, K.E., Cannizzaro, S.M., Lange, R.S., and Shakesheff, K.M. Polymeric systems for controlled drug release. Chem Rev, 99:1373-1379, 1999.

[5]. Frinking, P.A., Bouakaz, A.N., Folker, N.J., and Cate, T. Effect of ultrasound on the release of micro-encapsulated drugs. Ultrasound, 36: 709-713,1998;

[6]. Huang, S.L., and MacDonald, R. Acoustically active liposomes for drug encapsulation and ultrasound-triggered release. Biochimica Et Biophysica Acta, 1665: 134-141, 2004.

[7]. Rapoport, N., Marin, A., and Christensen, D. Ultrasound activation miceller drug delivery. Drug Deliv Sys Sci, 2 : 37-46, 2002.

[8]. Alexander, M., Muniruzzaman, M., and Rapoport, N. Mechanism of the ultrasound activition of micellar drug delivery. J Control Release,75:69-81,2000.

[9]. Rapoport, N. Combined cancer therapy by micellar-encapsulated drug and ultrasound. Int J Pharm, 277(1-2):155-162, 2004.

[10]. Brime, B., Ballesteros, M.P., and Frutos, P. Preparation and in vitro characterization of gelatin microspheres containing Levodopa for nasal administration. J Microencapsulation, 17: 777-784, 2000.

[11]. Hong, Wu., Zhenxi, Zhang., and Daocheng,Wu. Preparation and drug release characteristics of pingyangmicin-loaded dextran cross-linked gelatin microspheres for embolization therapy. J Biomed Mater Res part B: Applied Biomaterials, 78: 56-62, 2006.

[12]. Tian, Z.M., Wan, M.X., Lu, M.Z., Wang, X.D., and Wang, L. The alteration of protein profile of walker 256 carinosarcoma cells during the apoptotic process induced by ultrasound. Ultrasound in Medcine and Biology, 31: 121-128, 2005.

[13]. Tian, Z.M., Wan, M.X., Wang, S.P., and Kang, J.Q. Effects of ultrasound and additives on the function and structure of trypsin. Ultrasonics Sonochemistry, 11: 399-404, 2004.

[14]. Tian, Z.M., Wan, M.X., Wang, B., Wang, S.P., Wu,X.M., and Ruan, Y.S. Effects of ultrasound on the structure and function of tumor necrosis factor-α.. Ultrasound in Medicine and Biology, 29: 1331-1339, 2003.

[15]. Unger, E.C., McCreery, T.P., Sweitzer, R.H., Caldwell, V.E., and Wu, Y. Acoustically active lipospheres containing paclitaxel: a new therapeutic ultrasound contrast agent. Invest Radiol, 33: 886-892, 1998.

[16]. Young, S., Wong, M., Tabata, Y., and G. Mikos, A. Gelatin as a delivery vehicle for the controlled release of bioactive molecules. J Control Release, 109:256-274, 2005.

[17]. Husseini, G.A., Myrup, G.D., Pitt, W.G., Christensen, D.A, and Rapoport, N.Y. Factor affecting acoustically triggered release of drugs from polymeric micelles. J Control Release, 69:43-52, 2000.

[18]. Daocheng,Wu., Mingxi,Wan and Yinfon,Du. Enhancing the antitumor effect of adriamycin nanoparticle lipiodol emulsion by pretreating liver cancer with Diethyldithiocarbamate. Inter J Nonlinear and Numerical simulation, 3: 181-184, 2002.

[19]. Pharmacopoeia committee of Peoples Republic of China, Pharmacopoeia of the Peoples Republic of China. Chemistry Industry Press, Beijing, China, 2000.

[20]. Ghaleb, A., Husseini, G.D., Christensen, A., Rapoport, N., and Pitt, W.G. Kinetics of ultrasound release of doxorubicin from pluronic P105 micelles. Colloids and Surfaces B:Biointerfaces, 24: 253-264, 2002.