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Ultrasound-triggered thrombolysis using urokinase-loaded nanogels

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ABSTRACT

To find a way to modulate the effect of thrombolytic proteins by increasing their specificity, minimizing their adverse effect as well as lengthening their circulation time for the treatment of ischemic vascular disease holds great promise. In this work, urokinase-type plasminogen activator (uPA) was encapsulated into hollow nanogels which are generated by the reaction of glycol chitosan and aldehyde capped poly(ethylene glycol) (OHC-PEG-CHO) through a one-step approach of ultrasonic spray. The uPA-loaded nanogels, with size of 200–300 nm, have longer circulation time than that of the nude urokinase in vivo, besides the protein can be triggered to release in faster rate under diagnostic ultrasonic condition of 2 MHz, which significantly enhanced the thrombolysis of clots. The results are promising for increasing the specificity and positive effects of thrombolytic agents like recombinant tissue plasminogen activator (rt-PA) for the current treatment of ischemic vascular disease.

1. Introduction

Ultrasonic mediated targeting delivery systems are receiving increasing attentions in the passed decades especially for clinical treatments of emergency diseases (Tiukinhoy-Laing et al., 2007; Shaw et al., 2009; Smith et al., 2010; Uesugi et al., 2010). For example, stroke is the second leading cause of death and the first leading cause of disability in the world (Bonita et al., 2004; Feigin, 2005). Ischemic stroke accounts for about 3 quarters of all strokes for which intravenous recombinant tissue plasminogen activator (rt-PA) or urokinase-type plasminogen activator (uPA) remains to be the only treatment approved but must be done within a 4.5-h window (Hacke et al., 2008). However, due to the low specificity of rt-PA/uPA in systematic circulation, symptomatic intracerebral hemorrhage occurs to about 6.4% patients after an intravenous injection of thrombolytics according to the National Institute of Neurological Disorders and Stroke rt-PA Stroke Study (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995). As a result, the physicians are very prudent to use this kind of drug for a safety concern, meanwhile facing the fact that the proteins are suffering from fast clearance in vivo.

So far microbubble incorporated polymeric spheres and liposomes have been developed and the efficiency of those materials as ultrasound contrast agent and drug/gene carrier has been proven (Kimura et al., 1998; Tiukinhoy-Laing et al., 2007; Shaw et al., 2009; Smith et al., 2010). However the immunogenicity is remained as a major problem on the way of the microspheres to clinical trials, besides it comes to a safety issue for the injected gas, normally perfluoropropane, at high doses in case for therapeutical aims, no matter it has passed the FDA trial in imaging agents. In addition, smaller systems are more ideal particularly for the thrombolysis treatment because the thrombolitics have to be delivered to the thrombus through the narrow residual blood flow channels around the arterial obstruction (Blinc et al., 1994; Francis et al., 1995). Therefore, the development of new ultrasonic responsive nano- or submicrometer-sized drug carriers is still urgent.

Biodegradable polymeric nanoparticles have given much highlight as advanced vehicle of different kinds of drugs due to their advantages in terms of drug protection, transport and delivery (Panyam and Labhasetwar, 2003; Xu and Du, 2003; Ravi et al., 2004). With smaller size and desirable biocompatibility, polymeric nanoparticles can be transported through the blood circulation to remote body sites. Moreover, the nanoparticles are capable of responding to physical and/or chemical signals like pH, temperature as well as light by including sensitive chemical groups and polymer chains (Devine et al., 2006). These characteristics may favor the therapeutic applications in the delivery of thrombolitics for longer circulation time and the sensitivity to external stimuli. However the functionalization of the polymeric nanosystems with ultrasonic responsibility should be further addressed.

The objective of the current work is to use hollow nanogels, which were synthesized in our previous work (Zhao et al., 2011),

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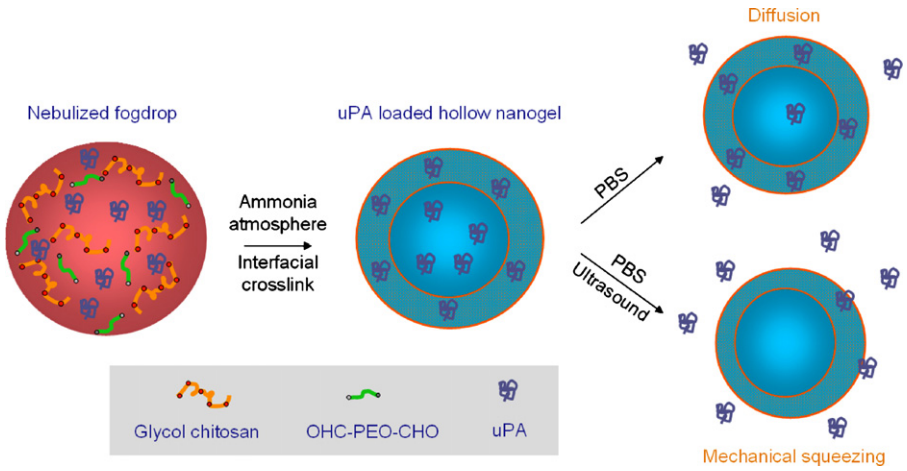

Scheme 1. Synthesis of ultrasound responsive uPA-loaded hollow nanogels via ultrasonic spray technique.

for ultrasound mediated delivery of thrombolytic proteins. With an inner cavity, the hollow nanogels were found not only having enhanced loading capacity of drugs, but also endowing a regional location of drug or diagnostic agents respectively in the cavity and the gel shell depending on the extent of interaction between the guest agents and the matrix, which leads to programmable drug release in response to environmental pH and temperature (Gu et al., 2007; Zhao et al., 2011). Herein we fabricate poly(ethylene glycol) (PEG) crosslinked glycol chitosan (GC) hollow nanogels through an ultrasonic spray technique, by which the thrombolytic agent, i.e. urokinase, can be simultaneously loaded (Scheme 1). The investigations proved that the hydrated hollow nanogels are sensitive to ultrasound within a safer diagnostic frequency range (2 MHz) that results in faster uPA release in buffer, without the inducement of gas in the matrix. This study may explore the potential application of soft nanoparticles to the ultrasonic sensitive drug delivery, as supported by the directed ultrasound thrombolysis gained by the uPA-loaded nanogels in this work.

2. Experimental

2.1. Materials and animals

Glycol chitosan (GC, MW = 250 kDa), 4-(dimethylamino) pyridine (DMAP) and dicyclohexylcarbodiimide (DCC) were purchased from Sigma–Aldrich (St. Louis, US). Benzaldehyde terminated polyethylene glycol (OHC-PEG-CHO, MW = 600 Da) was synthesized according to our previous report (Zhao et al., 2011). Urokinase-type plasminogen activator (uPA, MW = 49 kDa) was obtained from Livzon Pharmaceutical Group Inc., China. The bicinchoninic acid (BCA) protein assay kit was purchased from Ai De Lai Biotech Corporation, China. Chromogenic substrate of uPA (pyroGLU-GLY-ARG-pNA·HCl) and the analysis kit were obtained from Chemicon International Inc., USA. The human urokinase plasminogen activator enzyme-linked immunosorbent assay (ELISA) kit was purchased for the Cusabio Biotech Company, USA. Solvents and other compounds were obtained form Beijing Chemical Reagents Company, China.

Sprague Dawley (SD) male rats with weight of 200 ± 10 g were obtained from Vital River Company, China, and raised under normal conditions with free access to food and water. All care and handling of animals were performed according to the Guiding Principles for the Care and Use of Experiment Animals in Peking University First Hospital.

2.2. Preparation of the uPA-loaded GC/PEG hollow nanogels

Desired amounts of glycol chitosan, OHC-PEG-CHO as well as urokinase were dissolved in water at pH 5.0–5.5 (Table 1). The solution was pumped into a Sono-Tek ultrasonic nozzle with a peristaltic pump in a steady velocity. Ammonia was diluted by 50 times and sprayed through a bypath way in order to ensure a basic atmosphere around the fogdrops (Tan et al., 2012). The fogdrops were collected in a beaker and then transferred to a dialysis tube with a molecular weight cut-off of 12 kDa and dialyzed against $NaHCO_3$/NaOH solution (4.4 mM $NaHCO_3$, 1.4 mM NaOH, pH 8–8.5) at room temperature for 24 h with six changes, and then against distilled water with two changes in 8 h. The dialysate was freeze-dried to harvest white powder-like products with yields of 76–77 wt%, calculated from the mass of the product to the overall weight of feed reagents.

2.3. Characterization

Elemental analysis was carried out using a Flash EA 1112 NC Analyzers (Thermo Scientific, US). The GC and OHC-PEG-CHO composition in the nanogel products were determined by comparing the carbon/nitrogen (C/N) molar ratio to that of the parent polymers according to our previous report (Zhao et al., 2011). The size and zeta potential of the nanogel particles in aqueous solution was determined at 25 °C using a dynamic light scattering analyzer (DLS, Nano Series, Malvern Instruments, UK). The morphology of the nanogels was observed using scanning electron microscope (SEM, HITACHI S-4300, Japan) and the inner structure was viewed by transmission electron microscopy (JEOL 1011, Japan) on thin slice of the lyophilizated product. Fourier transform infrared spectroscopy (FTIR) was performed using a Bruker Equinox 55 spectrometer with the samples in KBr pressed pellets.

The loading capacity of uPA in the nanogels was determined using BCA protein assay. Briefly, known amount of uPA-loaded nanogels were dissolved in 1 mL of diluted hydrochloric acid solution (pH = 5.5) and stood for 30 min in order to dissociate the nanogels and release the protein to the aqueous phase (Zhao et al., 2011). The solution was infiltrated with a 100 nm membrane filter. 25 μL of the filtrate as well as uPA standards were pipetted into a 96-well plate. 200 μL of working solutions were added to each well

Table 1
Synthesis of uPA-loaded nanogels.

Total polymer concentration for spray (mg/mL)	Crosslinker feed ratio (—CHO/—NH_2, mol/mol)	Crosslinker content in the nanogels (mol% to GC segments)	uPA loading capacity (wt%)	Particle size (nm)		Zeta potential (mV)	
				As-prepared	After-sonication	As-prepared	After-sonication
0.5	0.2	7.5	52.8	211 ± 10	158 ± 8	1.98 ± 1.24	1.92 ± 0.39
1.0	0.2	9	51.8	348 ± 12	208 ± 11	0.77 ± 0.03	1.05 ± 0.21

followed by incubation at 37 °C for 30 min before the well plate was read on a microplate reader (SpectraMax M5, USA) at 562 nm. The mass concentration of urokinase hence the encapsulation capacity of the protein in the nanogels was determined according to the absorbance of the uPA standards. In case the encapsulated protein may diffuse into the aqueous phase during the dialysis in the synthesis procedure, the amount of free protein in the products was determined by filtering the water solutions of the nanogels (pH 7–7.2) through 100 nm membranes and filtrate was analyzed by the BCA assay. It was found that the uPA in aqueous phase was 0.5 and 2.8 wt%, depending on the polymer concentration for the nebulization, i.e. 1 mg/mL and 0.5 mg/mL (Table 1).

The influence of synthesis process on the bioactivity of the encapsulated uPA was determined on a chromogenic substrate analysis kit following the manufactures' introduction. uPA standards and the solution of testing samples, i.e. uPA and acid-dissociated nanogels with known mass of uPA, were pipetted into 96-well plates. The volume of the solutions in the wells was adjusted to be 180 μL by adding 20 μL of assay buffer (pH = 7.4) and sufficient amount of water. Then 20 μL of the chromogenic substrate solution (2.5 mg/mL) was added to the wells and the well plates were incubated at 37 °C. Optical density of the well plates at 405 nm was recorded periodically using a microplate reader (Versamax Tunable Microplate Reader). The bioactivity of uPA in the nanogel samples was calculated as activity units to mass ratio and compared with the nude uPA, which confirmed that the synthesis procedure has very limited influence on the bioactivity of the protein.

2.4. In vitro uPA release from the nanogels

1 mL of nanogel samples dispersed in PBS buffer (NaCl 136.9 mM, Na_2HPO_4 8.1 mM, NaH_2PO_4 1.9 mM) was pipetted into a 5 mL plastic pipe. The pipe was immersed and mounted in a static water bath to avoid the attenuation of the air and equilibrated at 37 °C. A diagnostic Transcranial Color Doppler (TCD) machine (VIASYS, UK) was equipped to the water bath with the transducer put in water 50 mm away from the plastic pipe. The frequency and output intensity were set at 2 MHz and 530 mW/cm^2, respectively. At a predicted time point, the solution was filtered using a 100 nm membrane filter and the urokinase concentration of the filtrate was detected by the BCA protein assay. Control experiments of release without the intervention of ultrasound were also carried out. The amount of the protein release was calculated as a mean value of three tests.

2.5. In vitro thromobolysis

Human blood was obtained from volunteers without any medication or hematological disease. 3 mL of blood was collected in non-anticoagulation tube (BD, Spain) and placed in a holder at 37 °C in water bath for 3 h to form the clot. To avoid the influence of the clot age, the initiation of thrombolysis was started at 5 h uniformed from the blood drawn. After absorbing the surface water with absorbent paper, the moist clot was divided into parts in cylinder morphology using a scalpel, with each part weighed 350 mg approximately. The clot cylinders were put into tubes containing 1 mL of urokinase PBS solution (3 mg/mL, pH = 7.4) or uPA-loaded nanogels (synthesized from the polymer solution of 0.5 mg/mL) with equivalent uPA units. The tubes were then immersed in static water bath at 37 °C with or without ultrasound intervention for desired periods. The clot mass loss was defined as an average value of $(W_0 - W_t)/W_0$ of three samples, where W_t and W_0 are the weights of the clots measured at predicted time points and the original weights of the clots respectively.

2.6. Pharmacokinetics of uPA-loaded nanogels and its biotoxicity in rats

Twelve SD male rats were randomly divided into 3 groups and intravenously injected 0.5 mL of PBS, uPA PBS solution and the PBS solution of uPA-loaded nanogels (synthesized from the polymer solution of 0.5 mg/mL) respectively. The administration level of uPA was 0.5 mg/rat (2000 units/rat) for the two corresponding groups. At 2, 5, 10, 20, 30, 60 and 120 min after the injection, around 0.5 mL of blood samples were collected from the caudal vein to the heparin-coated EP tube by cutting off the tail of the animals. Plasma was got by centrifugation at 3000 r/min for 1 min, and transferred into 1.5 mL EP tubes and stored at −20 °C before the uPA concentration was determined in an ELISA assay kit (Cusabio Biotech Company, USA). After 2 weeks of the injection, the mice were sacrificed with 3% barbital sodium at 1 mL/kg injected intraperitoneally. The hearts, livers, kidneys and brains were operated and fixed in 10% neutral-buffered formalin, then embedded in paraffin, sectioned at 4–6 μm and stained with hematoxylin and eosin (H&E) using standard techniques for histopathological examination, except the brains were stained with potassium hexacyanoferrate for the hemosiderin detection.

2.7. Statistical analysis

SPSS 13.0 statistical analysis software was used. Continuous variables were expressed as mean ± SD. Multiple groups were compared using the analysis of variance (ANOVA) with pairwise multiple comparison carried out using method of Student–Newman–Keuls (SNK). The difference was significant when the value of $P < 0.05$.

3. Results

3.1. Synthesis of the urokinase-loaded nanogels

The synthesis of GC/PEG nanogels using ultrasonic spray has been described in our previous work (Zhao et al., 2011). Benzaldehyde terminated PEG reacts with the amino group of the glycol chitosan in the nebulized fogdrops floating in basic atmosphere to form hollow nanogel particles via forming benzoic-imine linkages (Qu and Yang, 2011). With an aldehyde to amino group feed ratio of 0.2 mol/mol, the crosslinker content is 7.5–9 mol% to the GC segments, which is influenced by the polymer concentration for nebulization (Table 1). The crosslinking degree of the nanogels should be slightly lower than the OHC-PEG-CHO content since there would be small amount of the crosslinker molecules with only one end grafted to the GC backbones (Zhao et al., 2011). Fig. 1 proves the hollow structure of the nanogels. And Table 1 presents the mean particle sizes and zeta potential of uPA-loaded nanogels which demonstrates that spraying of polymer solution with higher concentration gained nanogels with larger size. Besides, the zeta potential is mild positive attributed to the surface distribution of PEG chains.

The uPA was in situ incorporated in the nanogels since being dissolved in the feeding solution of GC and OHC-PEG-CHO. The encapsulation rate is close to 80 wt% of total feed proteins under the experimental condition, as identified using BCA analysis, resulting in a loading capacity of ca. 50 wt% to the nanogels (Table 1). Previous work has demonstrated that hydrophilic nanoparticles are favorably localized in the gel shell of the hollow nanogels fabricated using the same technique, due to stronger interaction with the matrix (Zhao et al., 2011). In this work, this would also happen to the uPA because the protein could form hydrogen-bonding and electrostatic interaction with the hydroxyl and amino group of GC.

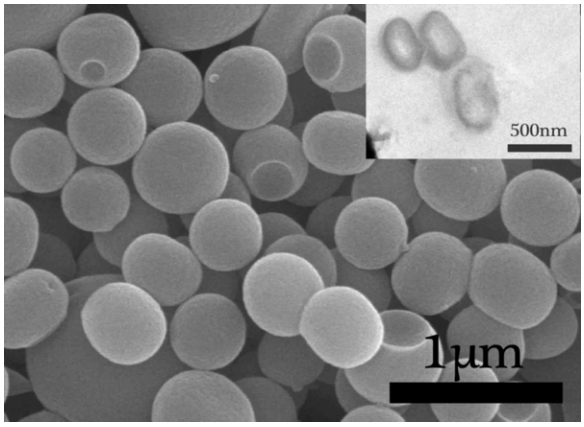

Fig. 1. The SEM of uPA nanogels prepared under a polymer concentration of 1 mg/mL and a crosslinker feed ratio (—CHO/—NH_2, mol/mol) of 0.2. Inset: TEM image of sliced nanogel particles.

Furthermore, the amino group of lysine residues in the protein may also react with the crosslinkers leading to chemically entrapments, although the extent was not determined. Nevertheless, circular dichroism (CD) tests showed no change of protein conformation in the nanogel compared to that of free uPA. This suggests that physically encapsulated proteins should be dominating (Tan et al., 2012), which can be explained by that the pK_a of the lysine residues (~9) is higher than that of GC (~6.5), implying that the imination between the crosslinker and GC is more favorable. In addition, it is also confirmed that the bioactivity of the encapsulated proteins was not influenced by the synthesis procedure, due to the dynamic nature of the benzoic-imine linkage and the mild condition of the synthesis (Tan et al., 2012).

3.2. The ultrasound mediated protein release of the uPA-loaded nanogels

The cumulative release of the uPA-loaded nanogels is plotted in Fig. 2. It demonstrates that without sonication, the release is under the Fickian diffusion mechanism, i.e. $n \sim 0.3$ (Fig. 2), and not significantly influenced by the synthesis parameters, i.e. the polymer concentration, hence the particle size of the nanogels, where around 80% of proteins were released within 6 h. Although it is considered that part of the protein molecules may be covalently bonded by the crosslinker during the preparation procedure, as a

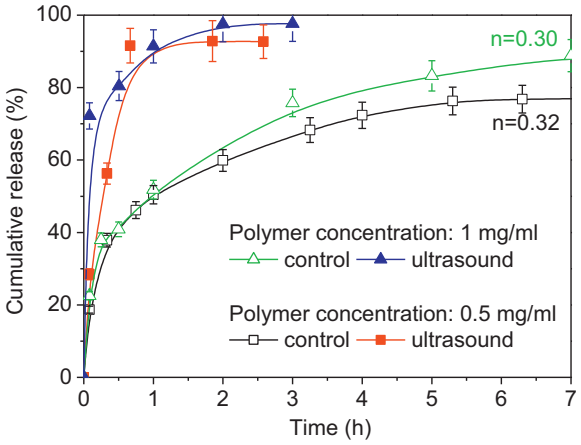

Fig. 2. Cumulative release of uPA from the nanogels. The release profiles are fitted using the equation of $M_t/M_\infty = kt^n$, where M_t/M_∞ is the fraction of drug release at time t, and n is the diffusion related component with a value varying from 0 to 1.

dynamic covalent bond, the imine linkage will hydrolyze while the uPA molecules are diffusing into the release media. Meanwhile, the stability of the nanogel matrix has been proven at physiological pH (Zhao et al., 2011).

The protein release is extremely accelerated upon the intervention of ultrasound, by which more than 90% of uPA was released within 1 h, as shown in Fig. 2. Right now, despite the microbubble encapsulated liposome systems, there are few reports on ultrasound mediated delivery by using vehicles without the inclusion of gas. Polymeric micelles such as formed by Pluronic P105 were recognized being responsive to ultrasound of kilohertz frequencies thus essentially through the cavitation mechanism (Husseini et al., 2000; Husseini and Pitt, 2008). However in the present work, it is found that the morphology of the nanogels is maintained in the TEM and SEM images (data not shown). Furthermore, the no obvious change of typical vibrations of polymer matrix, especially the imine absorbance, in FTIR spectrum is observed between the ultrasonic treated and untreated nanogels. The above results suggest that the structure of the matrix was not destroyed by the ultrasound power under the experimental condition. Meanwhile, DLS measurement shows that the particles size of the nanogels became smaller after sonication (Table 1), probably due to the release of protein molecules from the nanogel particles. The results therefore imply that the ultrasonic mediated release is driven under a mechanical deformation of the nanogels, probably because of the soft nature of the matrix after being fully hydrated. Nevertheless the mechanism especially the role of the hollow structure of the nanogels on the ultrasonic responsibility needs further investigation.

3.3. Pharmacokinetics and biotoxicity of uPA-loaded nanogels

The pharmacokinetics of the uPA loaded in the nanogels was evaluated on SD rats by ELISA on blood samples withdrawn from the tail vein. Fig. 3 presents the metabolic situations of the nude urokinase and the uPA-loaded nanogels as a function of time. Compared to the half life-time ($t_{1/2}$) of nude uPA, i.e. 18 min, the $t_{1/2}$ of the protein is significantly lengthened by the nanogels to ca.

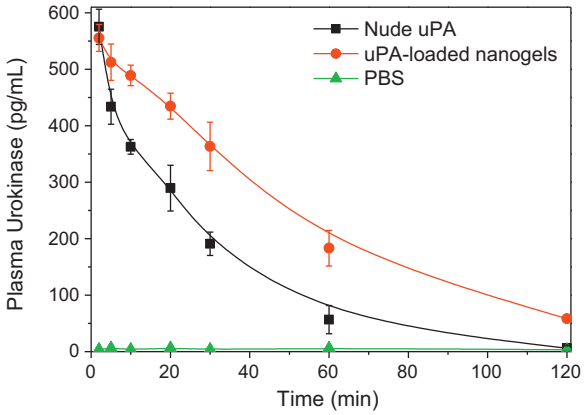

Fig. 3. The metabolic situation of the uPA and uPA-loaded nanogels in rats.

40 min. After a 2 h, the residue uPA concentration in blood is still ca. 60 pg/mL in the group of animal injected the nanogel formulation, whereas it becomes neglectable in the animals dosed by the nude uPA.

A two-week trial was applied on the biotoxicity of the protein loaded nanogels and the histological results are shown in Fig. 4. From the pictures of H&E stained liver, heart and renal tissues, no lesions were found in the animals after the administration of the nanogels, which proved the safety of the polymer matrix as well as the formulation of uPA with the nanogels.

3.4. Thrombolysis ability of the uPA-loaded nanogels

The thrombolysis capacity of uPA formulations is one of the most important characteristics that are closely related to the clinical applications (Holland et al., 2007; Hölscher et al., 2009). As shown in Fig. 5, it is found in the in vitro tests that the thrombolysis of uPA-loaded nanogels in silent condition is much lower than that of the nude uPA. In the first 30 min of incubation, 30 wt% of the clots was dissolved by nude uPA whereas it was 21 wt% by

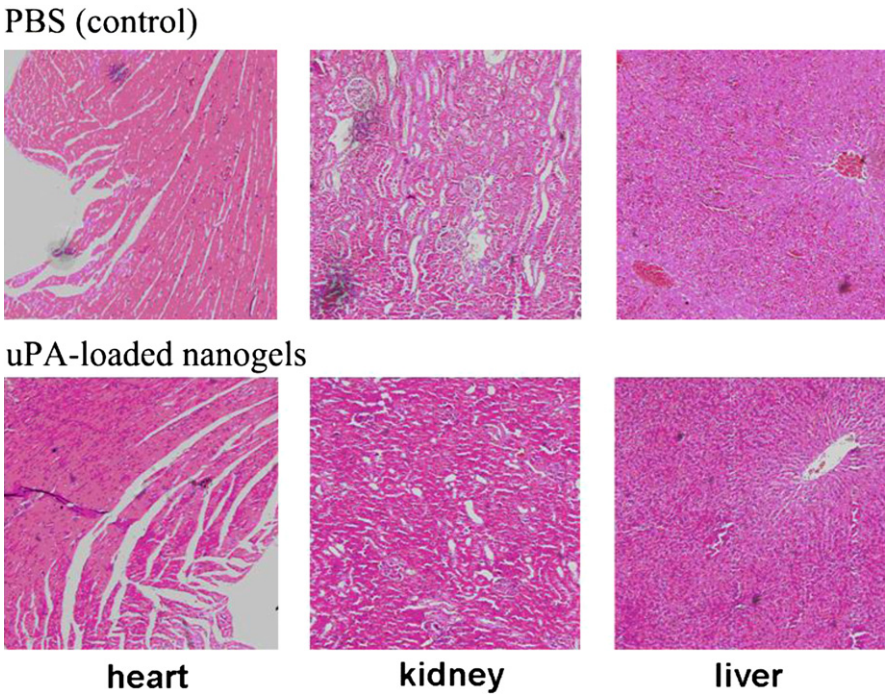

Fig. 4. H&E stained histological images of heart, liver and kidney of SD rats after administration of PBS or uPA-loaded nanogel dispersions for two weeks. Magnification 10×.

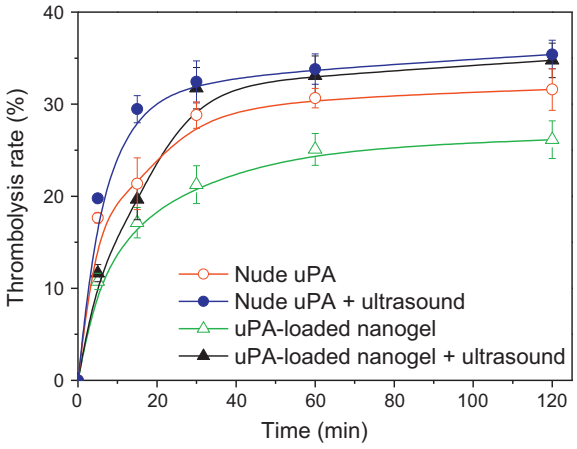

Fig. 5. Thrombolysis rate of uPA-loaded nanogels and nude uPA (control) with and without an ultrasonic intervention. The frequency and output intensity were 2 MHz and 530 mW/cm^2 respectively.

the nanogel containing equivalent amount of the protein, which again demonstrate the reserving ability of the nanogel matrix in the release study.

An ultrasonic intervention enhances the thrombolytics of clots for both nude uPA and the uPA-loaded nanogels, however under very different amplitudes (Fig. 5). Previous studies have shown that the application of ultrasound can enhance the thrombolysis of clots, due to the ultrasound looses the clot and allow the thrombolytics to diffuse into deeper regions (Amaral-Silva et al., 2011; Brown et al., 2011; Culp et al., 2011). However, the current work show that the effect of ultrasound on the thrombolysis by the nude uPA is rather limit, i.e. $P > 0.05$ vs. the silent thrombolysis at time points longer than 30 min (Fig. 5). In contrast, the effect of the ultrasonic intervention on the thrombolysis ability of the nanogel is significant, i.e. $P < 0.01$ after 30 min or longer compared to silent condition (Fig. 5). The results are in agreement with the in vitro release data of the nanogels, where a complete release of uPA was gained soon after a sonication while it keeps at a slow release rate under normal condition. Furthermore, under sonication, the equilibrated thromboylsis of the nanogels is similar to that gained by same amount of nude uPA (Fig. 5), which implies the complete release of uPA under sonication and again proves that the bioactivity of the uPA is well preserved in the nanogels.

3.5. Discussion

Protein based therapeutics is flimsy in vivo due to fast clearance by enzymes like proteinase. For instance, the half life-time of urokinase is only 10–15 min in human body (Erdogan et al., 2005). Thus protein drugs may have to be administrated at a high level close to the maximum tolerated dose to the patients in order to get a therapeutical concentration in the targeted site (Zaidat et al., 2002). However, such strategy is not applicable to many proteins like the rt-PA or uPA because an increased blood drug concentration of those drugs will cause serious side-effects such as hemorrhage (Brekenfeld et al., 2007). Therefore the site-specific delivery of the bioactives becomes an attractive way. In particular, ultrasound directed delivery has been investigated using microbubble based systems, in which there are still many unresolved questions to find the optimal combination for thrombolysis. Several investigators have utilized the frequency of kilohertz to accelerate rt-PA thrombolysis in vitro (Siegel et al., 2000; Akiyama et al., 1998), and recovered that a combination of rt-PA and an experimental kilohertz-delivery system resulted in an excessive risk of intracerebral hemorrhage and tissue injury (Daffertshofer and Hennerici, 2003). Besides, the clinical trial (TRUMBI) which has used low frequency ultrasound of 300 kHz was also stopped due to the significant increase in hemorrhagic events (Daffertshofer et al., 2005). On the contrary, both in vitro and in vivo models showed that a diagnostic ultrasound beam of 2 MHz pulsed-wave, as used in the present work, is safe when applied in the monitoring of the presence of obstructive intracranial thrombus and furthermore the thrombolysis evaluation during a treatment, and thus shows a promising outlook as an adjuvant for thrombolysis treatment (Daffertshofer and Hennerici, 2003; Alexandrov et al., 2004). Nevertheless, suitable drug carriers must have two basic features. Except a rapid responsibility to the ultrasound in the safe frequency range, the carriers should basically have a preservation property for the proteins within desired period, which would encourage a higher dose of thrombolytics to a larger portion of patients with ischemic stroke, while limit the occurrence of hemorrhage. Besides, the biocompatibility and biodegradability should be also concerned during the design of the delivery systems.

In this work, the nanogel particles are consisted of glycol chitosan and PEG, for which most of the safety issues have been investigated (Kim et al., 2009; Makhlof et al., 2010). The hollow structure constructed in the nanogel particles is expected to favor the drug loading (Gu et al., 2007). In addition, a gel shell crosslinked via benzoic-imine linkage is also sensitive to the environmental pH which has been exploited to the control of drug release kinetics (Qu and Yang, 2011). Nevertheless, this paper is considered as the first report on the investigation of ultrasonic mediated protein release from hollow nanogel systems. Since no gas was encapsulated into the particles, the mechanism of accelerating drug release is attributed to the mechanical force of ultrasound, though it should be further clarified in the future works. Anyway, the in vitro tests reveal that the proteins can be almost released from the nanogels within 1 h under sonication (Fig. 2), which resulted in the fast clot thrombolysis within the therapeutical window, i.e. less than 4.5 h from the symptom onset of ischemic stroke patients (Fig. 5). At the same time, the release of uPA from the nanogels under silent condition keeps at slower rate. The preservation ability of the nanogels will not only benefit the protection of the protein from fast biodegradation hence prolongs the circulation as proved in vivo (Fig. 4), but also prohibit the bioactivity of the protein to non-specific sites, as evidenced by the lower thrombolysis under normal condition (Fig. 5a), which will significantly reduce the risk of acute hemorrhage complication. In contrast, the effect of ultrasonic intervention on the thrombolysis of nude uPA is eventually not obvious due to the similar level of thrombolytic performance when without sonication (Fig. 5b). This suggests that the hollow nanogels are promising as ultrasounic responsive delivery systems to diagnostic ranging pulsed-waves.

It was noticed that the nanogels could not preserve encapsulated proteins from diffusing into the aqueous phase under silent condition (Fig. 2). However the release kinetics of the formulation is expected to be adjusted by tuning the microstructure of the nanogels, for example, via adjusting synthesis parameters to vary the crosslinking degree and the chemical composition of the shell. Nevertheless, previous studies showed that besides a rush dose of high level plasminogen activators, a prolonged dose under a safe level would be also insufficient because of the urgent requirement for the recanalization such as during the arterial reocclusion treatment, in which the first remarkable improvement of flow to the brain is reported to occur no later than a median time of 17 min for rt-PA bolus (Alexandrov et al., 2001; Hacke et al., 2004). Therefore, it becomes clinically significant that the nanogel carrier in our work could maintain ca. 75% of dosed protein in circulation at 20 min after injection, as shown in the rat model (Fig. 3). Moreover, besides the physiological protection of the protein, the carrier has smaller size than most of the liposome systems and a PEG covered surface which

could inhibit the immunology response from the reticuloendothelial system (Hu et al., 2003; Kim et al., 2009). The histological tests observed no hemorrhage or hemosiderin in the tissue of brain, nor hemorrhagic complication in the group of animals after treated by the nanogels.

4. Conclusions

Urokinase-loaded hollow nanogels were synthesized via a one-step spray-drying route, and proved to be responsive to the 2 MHz diagnostic ultrasound, which will favor the application as a safe and effective vehicle for bioactives such as the thrombolytic agents. Our in vitro and in vivo investigations in this work demonstrate that the life-time of uPA has been significantly prolonged in rat model upon the protection by the nanogel matrix while the thrombolysis rate of blood clots can be efficiently accelerated through the intervention of ultrasound. Further work to evaluate this new material with ischemic stroke animal models is warranted for lighting future clinical sonothrombolysis study for patients with ischemic stroke.

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