



# In vivo evaluation of urokinase-loaded hollow nanogels for sonothrombolysis on suture embolization-induced acute ischemic stroke rat model

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## ABSTRACT

The urokinase-type plasminogen activator (uPA) loaded hollow nanogels (nUK) were synthesized by a one-step reaction of glycol chitosan and aldehyde capped poly (ethylene oxide). The resultant formulation is sensitive to diagnostic ultrasound (US) of 2 MHz. Herein, we evaluated the in vivo sonothrombolysis performance of the nUK on acute ischemic stroke rat model which was established by suture embolization of middle cerebral artery (MCA). Via intravenous (i.v.) administration, the experimental data prove a controlled release of the therapeutic protein around the clots under ultrasound stimulation, leading to enhanced thrombolysis efficiency of the nUK, evidenced from smaller infarct volume and better clinical scores when compared to the i.v. dose of free uPA no matter with or without US intervention. Meanwhile, the preservation ability of the nanogels not only prolonged the circulation duration of the protein, but also resulted in the better blood-brain barrier protection of the nUK formulation, showing no increased risk on the hemorrhagic transformation than the controls. This work suggests that the nUK is a safe sonothrombolytic formulation for the treatment of acute ischemic stroke. © 2017 The Authors. Production and hosting by Elsevier B.V. on behalf of KeAi Communications Co., Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 1. Introduction

Stroke is an important cause of death and disability around the world [1]. Thrombolysis and endovascular interventional therapy are both evidence-based therapy for vascular recanalization [2]. Except the recombinant tissue plasminogen activator (rt-PA), urokinase-type plasminogen activator (uPA) is the second thrombolytic agent used in clinical practice, especially in the primary

stroke center in China [3,4]. For thrombolytic action, uPA enables to active the fibrinolytic system to catalyze the production of plasmin for fibrin-splitting while it can cause the degradation of fibrinogen, Factor V and Factor VIII to inhibit the occurrence of thrombin. Besides, it was also found that uPA is positive to increase the activity of ATPase, and hence hampers the ADP mediated platelet aggregation which decreases the tendency of thrombogenesis [4].

The treatment of cerebral ischemic stroke using rt-PA or uPA is

*Abbreviation:* SD, Sprague-Dawley; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; UK, urokinase; nUK, uPA-loaded nanogels; US, ultrasound; UK+US, ultrasound and free urokinase; nUK+US, ultrasound and uPA-loaded nanogels; TCD, Transcranial Doppler; MRI, magnetic resonance imaging; EB, Evans blue; HT, hemorrhagic transformation; Hb, hemoglobin; BBB, blood-brain barrier; ELIP, echogenic liposomes; CCA, common carotid artery; TTC, 2,3,5-triphenyltetrazolium chloride.

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normally limited by the narrow time window as well as the risk of hemorrhage complication. As a protein, either rt-PA or uPA suffers from the shorter circulation duration due to the fast clearance by the proteinase *in vivo*. These facts could have led to the poor clinical outcome of patients with large artery occlusion, for example, a low recanalization level around 40% at twenty-four hour after an intravenous (i.v.) administration of the thrombolytic enzymes [5–7]. Furthermore, even the major occluded brain arteries were recanalized, patients may still fail to achieve clinical improvement since the ischemic tissue might not benefit from the recanalization due to the clots in the microcirculation and injuries to the endothelium [8,9], which makes microcirculation no-reflow, a phenomenon in which major vessel recanalization does not result in some part of microvascular reperfusion [9–11]. However, increasing the dose level would not be completely accepted in clinical application for better thrombolysis due to the neurotoxicity of the proteins [10], besides the concern of hemorrhage.

The improvement of specificity of the thrombolytic agents is a way to overcome the drawbacks of the rt-PA or uPA treatment. Many studies showed that entrapment of the proteins into a delivery system like liposomes could benefit the thrombolysis primarily resulting from the prolongation of circulation duration and the capacity of endowing passive or active targeting ability [12–20]. Among them, ultrasonic (US) induced delivery of thrombolytic proteins using microbubbles has demonstrated promising properties for site-specific release of payloads from the carrier through cavitation effects and acoustic radiation force on the carrier [21]. Besides, recent researches also showed that with microbubbles, ultrasound could not only recanalize the acute intravascular thrombi in large vessels [22–27], but also make the microcirculation recanalization [28–31], showing accelerated thrombolysis via the generation of localized mechanical stress on the thrombi [32]. Actually, the ability of ultrasound (US) energy to force enzymatic thrombolysis was already described in 1976 [33] and several experimental studies have confirmed this finding [34–36].

Nevertheless, to date, neither sonothrombolysis with nude intravenous thrombolytics nor with intravenous administering formulations, e.g. thrombolytic loaded echogenic liposomes (ELIP), had acknowledged ultrasonic parameters and material consistency. Moreover, there are reports to suspect that with an ultrasonic frequency of 300 kHz, the thrombolysis process may take enhanced venture on intracerebral hemorrhage [37,38]. On the other hand, the utility of higher frequency ultrasound is considered safer [39–41]. In previous work, we synthesized hollow nanogels (diameter ~ 200 nm) for the loading of uPA [17,42]. The nanogels composed of glycol chitosan (GC) and benzaldehyde-capped polyethylene oxide (OHC-PEO-CHO) were fabricated via an one-step procedure for crosslinking through *in situ* Schiff's reaction. We found that the nanogels enabled to respond the ultrasound at 2 MHz, a frequency being already used in diagnosis of intracranial artery stenosis. And different from microbubbles, the nanogels contains no gas. The responsibility was attributed to the vibration of the crosslinked polymer matrix driven by the ultrasonic energy. *In vitro* experiment has shown that under the ultrasonic mediation, the cargoes, i.e. the loaded uPA molecules, could be favorably released to enhance the thrombolysis of clots while *in vivo* tests proved that the half-life time of uPA in circulation system was significantly prolonged [17]. Although such characteristics indicate promising potentials for the uPA loaded nanogels to be used in stroke treatment, especially for safety issues, the test of real thrombolytic effect in animal model is still necessary. Therefore, in this study we established persistent middle cerebral artery occlusion (MCAO) model on Sprague-Dawley (SD) rats to investigate the efficiency and mechanism of combining uPA with ultrasound intervention by the hollow nanogel carrier for the thrombolysis of

acute ischemic stroke.

## 2. Material and methods

### 2.1. Animal model

The animal studies were approved by the Animal Research Committee of Peking University First Hospital (Beijing, China), and the investigation conformed to the Guide for the Care and Use of Experiment Animals of Peking University First Hospital. Adult male SD rats weighing 270–300 g were obtained from the Vital River Company, China, and raised under normal conditions with free access to food and water. The animals were anesthetized with pentobarbital at a dose of 50 mg/kg through intraperitoneal injection and then subjected to permanent MCAO by use of the filament model as previously described [43,44]. Briefly, the right common carotid artery (CCA) were ligated permanently and a silicon-coated 6-0 surgical monofilament nylon suture 4 cm in length was advanced proximally until mild resistance was felt to occluding blood flow to the right MCA. The signs of a successful surgery included the following: flexion or less grasping ability of the left foreleg and spontaneous circling or toppling to the left. For transcranial ultrasound application, being similar to the previous studies [44,45], the animals were shaved on the scalp. And a plastic ring filled with ultrasonic coupling gel (diameter 3.5 mm, 10 mm) was placed on the midline of the animal's head. A diagnostic Transcranial Doppler (TCD) machine (VIASYS, UK) with the transducer was placed 5 mm above the skull to ascertain the full transmission of sound to the skull. The frequency and output intensity were set at 2 MHz and 530 mW/cm<sup>2</sup>, respectively.

### 2.2. Preparation of the uPA-loaded hollow nanogels (nUK)

Benzaldehyde terminated poly(ethylene oxide) (OHC-PEO-CHO, MW = 600 Da) and uPA-loaded glycol chitosan (GC)/OHC-PEO-CHO hollow nanogels were synthesized according to our previous report [42]. GC, OHC-PEO-CHO and urokinase were dissolved in water at pH 5.0–5.5. 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 fog drops. The fog drops 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<sub>3</sub>/NaOH solution (4.4 mM NaHCO<sub>3</sub>, 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 wt %, calculated from the mass of the product to the overall weight of feed reagents. The morphology of the nanogels was characterized using scanning electron microscope (SEM, HITACHI S-4300, Japan) and transmission electron microscopy (JEOL 1011, Japan). And the loading capacity of the uPA was measured using BCA protein quantitative kit. The uPA loaded nanogel (nUK) products were stored at –20 °C before use.

### 2.3. *In vivo* thrombolysis

The rats with signs of successful surgery were divided into five groups with each group of 42 rats as: group 1, permanent MCAO with saline treatment (denoted as MCAO); group 2, MCAO with UK treatment (UK); group 3, MCAO with nUK treatment (nUK); group 4, MCAO with US plus UK treatment (US+UK); group 5, MCAO with nUK plus US treatment (nUK+US). The active urokinase dose was 100,000 U/kg in the UK and nUK groups and was applied through intravenous tail injection 30 min after MCAO. And the 2 MHz US

was applied 1 min after the injection with the procedure as described above and performed for 30 min. The equivalent volume of saline was injected into control group with similar procedure.

#### 2.4. Measurement of uPA in the peripheral blood

The blood sample was drawn from caudal vein at 2, 5, 10, 15, and 25 min after the injection of the formulations and centrifuged at 3000 rpm for 15 min. The concentration of uPA was detected by uPA ELISA kit (Cusabio, Wuhan, China) according to the manufacturer's instructions.

#### 2.5. Assessment of neurological deficits

At 24 h after embolization, the rats were evaluated for clinical signs of stroke and scored as follows [46]: 0, no paralysis; 1, flexion of contralateral foreleg; 2, less grasping ability of the left foreleg when lifted up; 3, circling when pulled; 4, circling spontaneously; and 5, disturbance of consciousness and death.

#### 2.6. Magnetic resonance imaging and 2,3,5-triphenyltetrazolium chloride staining of the brain

Cerebral magnetic resonance imaging (MRI) was performed in rats 24 h after surgery, the T2-weighted MRI images of the whole mice were measured from a 7T small animal MRI system (7T MRI System, Agilent, USA). The axial images were obtained with the TE (echo time) of 72 ms, TR (repetition time) of 3000 ms, the slice thickness of 1 mm and the matrix of  $256 \times 256$ .

The brains were removed and frozen in a  $-20^\circ\text{C}$  refrigerator for 15 min. Then the entire brain was cut into six 2-mm slices for 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) staining [43]. The total infarct area of each slice was recorded as A ( $\text{mm}^2$ ). The area of the intact ischemic hemisphere of each slice was recorded as B ( $\text{mm}^2$ ). To avoid error introduced by brain edema, infarct volumes were calculated using an indirect method [47]. In brief, the area of the intact contralateral hemisphere of each slice was recorded as C ( $\text{mm}^2$ ), and the thickness of every layer was recorded as D (mm). The relative infarct volume (E,  $\text{mm}^3$ ) of each slice was calculated as  $E = [A - (B - C)] \times D$ . The total relative infarct volume was the sum of the E values.

#### 2.7. Measurement D-dimer and evaluation of Evans blue (EB) leakage

To evaluate the dynamic levels of D-dimer, peripheral blood was drawn through the angular vein plexus at 1, 2, 6, 10 and 24 h after treatment. Plasma was got by centrifuged at 3000 rpm for 15 min and the D-dimer concentrations were determined with commercial kits (Cusabio, Wuhan, China) following the manufacturer's instructions.

To examine the permeability of the blood-brain barrier (BBB), rats were injected intravenously with 2% (w/v) EB (Sigma) through the tail vein at 40 mg/kg one day after surgery. 60 min after the injection, the rats were anesthetized and transcardially perfused with saline until the outflow fluid from the right atrium was clear. After perfused, their brains were dissected, weighed and incubated in formamide solution in  $60^\circ\text{C}$  water bath. After 24 h, formamide solution was collected. Then the EB concentration of the formamide solution was measured using a microplate spectrophotometer (BioTek synergy 4, USA).

#### 2.8. Concentration of hemoglobin (Hb) of the brain tissue

To examine the hemorrhagic transformation (HT), the

concentration of Hb in the brain tissue was determined as previously described [43]. On day 1, 3 and 7 after MCA occlusion, the rats were anesthetized and transcardially perfused with saline until the outflow fluid from the right atrium was clear. Brain tissues were dissected out, homogenized, and centrifuged at 12,000 rpm for 15 min. Concentration of Hb in the supernatant was determined using specific detection kits (QuantiChrom™ Hemoglobin Assay Kit, Applied Biosystems, Inc, Carlsbad, CA, USA), following the manufacturer's instructions. The optical density (OD) at 400 nm was measured using a microplate spectrophotometer (BioTek synergy4, USA). The concentration of Hb was expressed as mg/dl tissue.

#### 2.9. Hematoxylin and eosin staining

To evaluate the systemic side effects of nUK, seven rats from each group were perfused with 4% (w/v) paraformaldehyde (Sigma) after 2 weeks of the treatment. Their heart, liver, kidney were removed, embedded in paraffin, sectioned at  $5\ \mu\text{m}$  and stained with hematoxylin and eosin (H&E) for routine evaluation of histology and inflammation.

#### 2.10. Statistics

Statistical analyses were performed using SPSS 16.0 software. The crosstabs were used for evaluation of death rates and HT. One-way analysis of variance (ANOVA) was used for evaluation the differences among the five groups if the data exhibited a normal distribution and homogeneity of variance. Otherwise, the

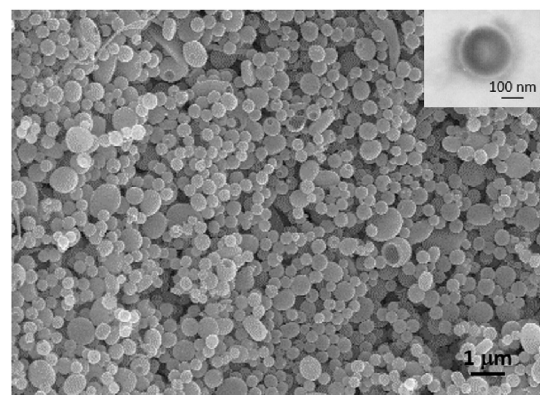


Fig. 1. SEM and TEM (inset) images of the synthesized uPA loaded hollow nanogels.

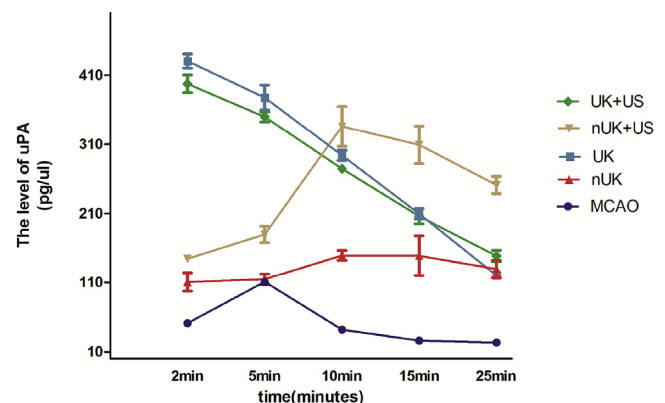


Fig. 2. Peripheral blood concentration of uPA in stroke rats after the formulations were administrated via i.v. injection.

differences were assessed by a non-parametric test. The comparisons of dynamic concentration changes of uPA and D-dimer were conducted through repeated measures analysis. If the results indicated significant differences, comparisons between two groups

were performed by post-hoc analysis using the least significant differences (LSD) *t*-test. For all statistical analysis, significance was accepted as  $p < 0.05$ .

### 3. Results and discussion

#### 3.1. Pharmacokinetics of nUK

The morphology of the nUK is shown in Fig. 1. The SEM displays sphere-shape particles and the TEM demonstrates the hollow structure of individual nanogel, i.e. a cavity surrounded by polymer matrix. The uPA loading capacity of the nanogel formulation is 52 wt% to the weight of the nanogel matrix. These characteristics are consistent to our previous report [17].

The pharmacokinetics of uPA in each group is shown in Fig. 2. It shows that the blood concentration of uPA in the free uPA treated rats (UK) was at the highest level sooner after the injection, i.e. 2 min, despite the intervention of US, but decreased rapidly within 25 min. In contrast, the administration of nUK resulted in much lower blood concentration of uPA in the rats throughout the monitoring period, once the US was not applied, an indication of well-preservative capacity of the nanogel carrier. However, with the application of US, the concentration of uPA was dramatically increased. Fig. 2 reveals that while the nUK+US group exhibits a low level of uPA similar as that in the nUK group in the initial stage,

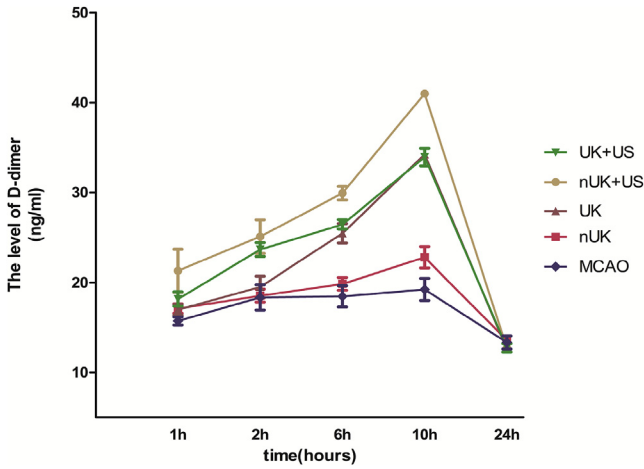


Fig. 3. The dynamic levels of D-dimer in the rats (Statistics: 6 h,  $P_{nUK+US \text{ vs } UK} = 0.021$ ;  $P_{nUK+US \text{ vs } UK+US} = 0.05$ ; 10 h,  $P_{nUK+US \text{ vs } UK} = 0.009$ ,  $P_{nUK+US \text{ vs } UK+US} = 0.010$ ).

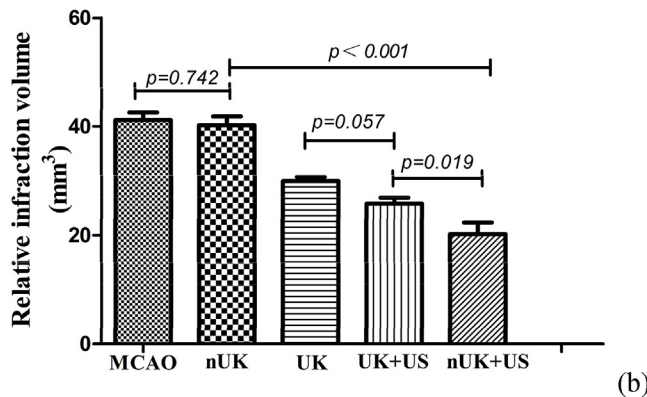
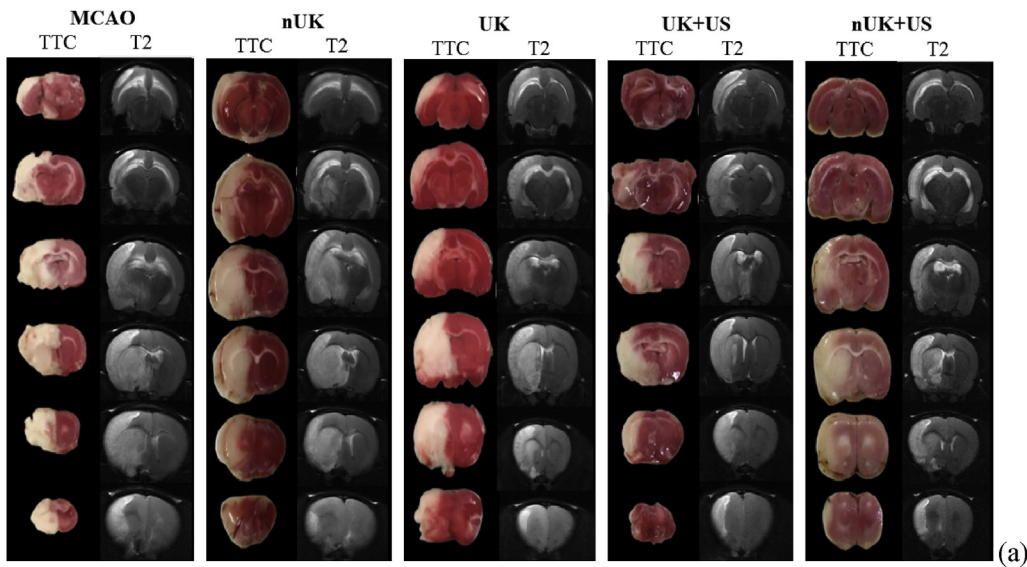


Fig. 4. (a) Representative TTC and T2 images on the infraction area where the infraction brain tissue was stained in white. (b) Relative infraction volume after the treatment.

i.e. 2 min, the protein concentration started to increase at next checking point, i.e. 5 min, following introduction of the US stimulation started at 1 min after the injection. The maximum uPA level was detected at 10 min after injection for the nUK+US group, more than two fold higher than the nUK group. After that, the uPA concentration in the nUK+US group is significantly higher than the other groups until the end the test, i.e. 25 min ( $p < 0.05$ ).

### 3.2. Clots degradation

The dynamic changes of clot degradation in the animals were evaluated by monitoring the produced D-dimer concentration in blood in a 24 h period and the results are shown in Fig. 3. While the D-dimer concentration keeps at ca. 20 ng/mL and shows no significant differences between MCAO and nUK groups at each time points, the level increases for the UK, UK+US and the nUK+US groups within the first 10 h. This confirms the action of the uPA on the thrombolysis and meanwhile means that the inducement of US caused significant difference in the performance of the nUK, by a US triggered release of the protein. On the contrary, free uPA is active without the US stimulation which led to high D-dimer production in the UK group, having no statistical difference to that of the UK+US group at 6 and 10 h. And it should be pointed out that the D-dimer level of the nUK+US group has obviously higher than that of the UK and UK+US groups ( $p \leq 0.05$ ) (Fig. 3).

### 3.3. Clinical outcomes

The relative infarct volumes were detected by 2,3,5-triphenyltetrazolium chloride (TTC) staining and MRI scan after 24 h of treatment (Fig. 4a). Among the formulations, in case there was no US assistance, the administration of nUK led to no positive effect to the MCAO, gaining a high infarct volume similar to that of the control group, i.e. MCAO ( $40.19 \pm 3.96 \text{ mm}^3$  for nUK vs.  $41.13 \pm 3.46 \text{ mm}^3$  for control,  $p = 0.74$ ), whereas the free uPA (UK) could efficiently decrease the infarct area ( $31.78 \pm 3.46 \text{ mm}^3$ ,  $p < 0.001$  vs. control) (Fig. 4b). This is reasonable because the pharmacokinetics study has demonstrated the difference of blood uPA concentration between the two uPA formulations (Fig. 2). And also as expected, the application of US improved the thrombolysis on the rat model, which significantly shrunk the infarct volumes of the animals in the nUK+US group ( $20.20 \pm 5.14 \text{ mm}^3$ ), although the difference between the UK and UK+US groups are not significant ( $31.78 \pm 3.46 \text{ mm}^3$  for UK vs.  $25.78 \pm 2.78 \text{ mm}^3$  for UK+US,  $p = 0.057$ ). The nUK+US harvested significantly better clinical outcomes, getting an average infarct volume of 20% smaller than that of the UK+US group ( $p = 0.019$ ) (Fig. 4b).

As a result, the clinical scores were significantly improved by the nUK+US, i.e.  $1.89 \pm 0.85$ , much better than the administration of either nUK ( $3.54 \pm 0.90$ ,  $p < 0.001$ ) or UK+US ( $2.47 \pm 1.05$ ,  $p = 0.03$ ) (Fig. 5). These results not only indicate the necessary of sonication for the treatment for the nUK, but also infer that with the carrier, the nUK formulation enables to improve the activity of the uPA for the thrombolysis compared to the free uPA (UK) under ultrasonic stimulation.

### 3.4. BBB protection

The concentration of EB was measured for showing the BBB integrity. Similar to the order of the infarction volume and the clinical scores (Figs. 4 and 5), the concentration of EB is at high level in the control and the nUK groups, i.e.  $11.18 \pm 2.51 \text{ } \mu\text{g/g}$  (MCAO) and  $11.03 \pm 2.10 \text{ } \mu\text{g/g}$  (nUK) respectively, while the level is significant lower in the free UK and ultrasonic treated groups ( $p < 0.001$ ) (Fig. 6a). However, among the three groups, i.e. UK, UK+US and

nUK+US, the EB concentration in the nUK+US group again gained statistically lower value than the other two groups ( $p = 0.031$ ), which means that the BBB was well-protected during the thrombolysis process when using the nUK formulation even though the treatment was promoted by the application of ultrasonication. On the other hand, it is seen in Fig. 6b that the concentration of Hb in the brain has no significant difference among each group including the MCAO that was examined at 1, 3 and 7 days. The results suggest that the administration of nUK formulation would not increase the risk of brain hemorrhage to the patients although the nanogel carrier is sensitive to ultrasonication by which the uPA molecules were triggered to release by mechanical stress.

### 3.5. Biocompatibility of nUK

To further check the biocompatibility of the nUK formulation, observations on hemorrhagic transformation (HT) and the survival rate were performed. From the perspective of pathology, Fig. 7a and

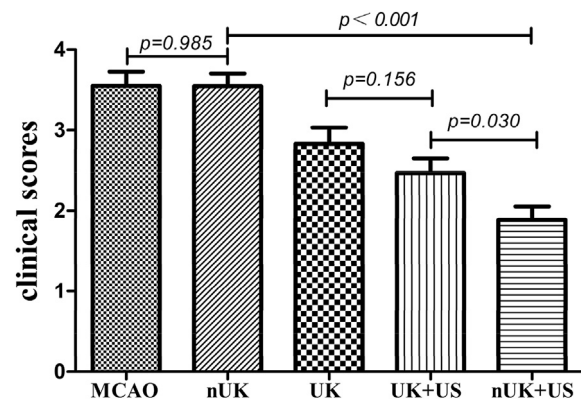


Fig. 5. The clinical scores of rats treated by different formulations.

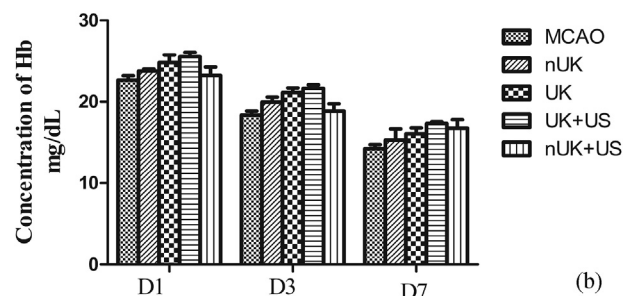
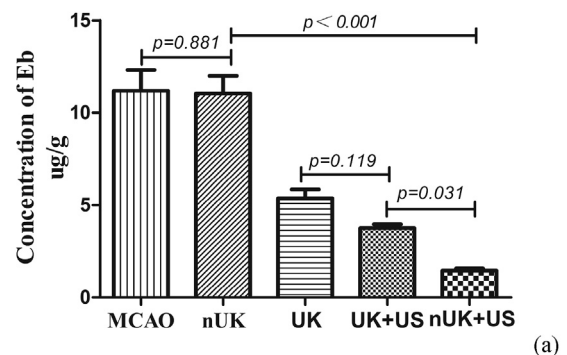


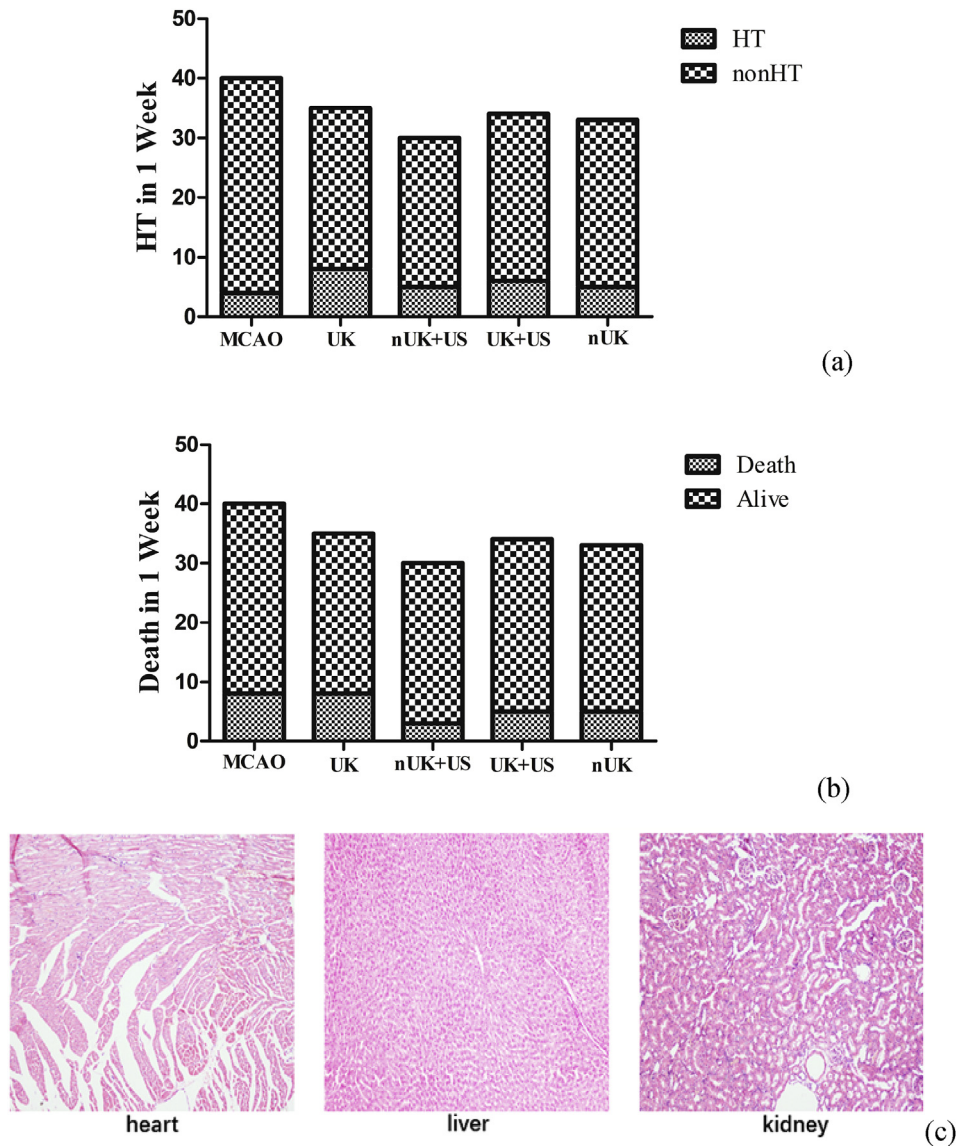
Fig. 6. (a) Concentration of EB in rats after treated by different formulations 30 min post-MCAO occlusion. (b) The concentration of Hb in the brain tissue on days 1, 3 and 7 after MCAO occlusion. No statistic difference among the formulations on each day in (b).

b shows that the administration of free uPA led to HT of 8 animals over the total number of 35 rats within one week of intervention, and eventually caused the death of 8 animals after one week. The UK+US group had 6 HTs and 5 death over 34 animals. Comparably, the MCAO and nUK groups gained HT and death number of 4 and 8 over 40 animals (MCAO), and 5 and 5 over 33 animals (nUK), respectively, while the nUK+US group demonstrates a HT of 5 and a death number of 3 over the total 30 rats. Statistics analysis shows no significant difference on both HT and the survival of the animals among the groups, which once again proves that either carrier or the application of ultrasound at the particular frequency, i.e. 2 MHz, would not cause extra harmful effect to the animals.

An additional two-week trial was then applied on the biotoxicity of the protein loaded nanogels (nUK) to other organs under ultrasonic stimulation and the histological results were shown in Fig. 7c. By H&E staining, the pictures show no lesions in heart, liver and renal tissues, which confirmed the safety of the nanogel formulation.

### 3.6. Discussion

Due to the concept of “time is brain”, early reperfusion therapy is essential for the ischemic tissues. Intravenous administration of thrombolytic is still the convenient and time-saving approach in the clinical practice. However, the complete recanalization rate is relatively low, especially for the total occlusion of large cerebral vessels [48–50]. In this case, the delivery of the thrombolytics via collateral circulation to canalize the microcirculation becomes important to reduce the damage of the brain [30,31]. Due to the low specificity of the thrombolytic, the risk of brain bleeding was the most concerning problem puzzling the clinicians for higher dose level [37,51]. Thus, targeted delivery of the thrombolytic to the clots is required to enhance its thrombolysis effect. Ultrasound was combined with thrombolytic agents for improving the thrombolysis treatment which has been studied both in vitro and in vivo, despite the inclusion of microbubble or not [17,35,51–53]. Multiple animals and clinical experiments showed that alteplase or



**Fig. 7.** HT (a) and survival rate (b) of different groups of animals after treated for 1 week. (c) H&E staining on heart, liver and kidney sections isolated two weeks after the treatment by nUK.

urokinase with ultrasound intervention could achieve smaller infarct volume [54,55] and got higher therapeutic efficiency than the use of thrombolytic alone [16,56,57]. Nevertheless, further development of ultrasonic responsive carrier is still attractive because a triggered release of the thrombolytic proteins will certainly increase the specificity of the delivery by the control of the external stimuli, which is expected to lower the risk of hemorrhage or to give the choice to increase the dose level [58].

Except microbubbles, our previous research showed that polymeric nanogels with a hollow cavity, without the inclusion of gas, e.g. perfluoropropane, are also able to respond the ultrasonic stimuli to release the cargoes more rapidly [17]. The mechanism for the ultrasonic sensitivity of the GC/OHC-PEO-CHO hollow nanogel is different from the cavitation effect induced by the sonoporation that happens to the microbubbles, but was attributed to the mechanical vibration of the gel shell. This might be a reason that resulted in the responsibility of the nanogels to higher ultrasound frequency of 2 MHz. Meanwhile, compared to liposomes, polymeric carriers are considered more stable against the dilution in the circulation systems and hence will have better preservation property to allow the transportation of payloads to promote site. Although the US triggered release of uPA from the nanogels has been investigated *in vitro* in the previous study, whether such a formulation enable to favor the thrombolysis therapy in a clinical model was not yet clarified which is the main objective of this work.

Early studies reported that the infarct volume of a 300 g rat after 2 h of MCAO could be 400–450 mm<sup>3</sup> which is close to the infarct volume of 24 h after MCAO occurrence in the animal, with roughly 140 mm<sup>3</sup> brain tissue dead in the first 30 min of ischemia, and about 90 mm<sup>3</sup> dead in next 30 min [59]. Therefore, the MCAO rat model was used after 30 min of ischemia in our study to simulate the arterial thrombolysis within the therapeutic time window. However, it should mention that with MCAO induced by a suture embolization approach, the permanent ligation of the CCA would cause irreversible ischemia of the involved brain segments despite the use of thrombolytics. Herein, the *in vivo* results confirm that the application of US is essential for the nUK formulation to exhibit thrombolysis ability on the rat model, which implies the recanalization of the microcirculation upon the combinatorial action of the therapeutic protein and the ultrasonic field. Without the application of US, the nUK showed negative therapeutic effect whereas it displayed significant clinical outcomes than the free uPA whenever under ultrasound intervention. This is majorly contributed by the controlled release property of the hollow nanogel carrier, which well-preserved the uPA molecules in the matrix. Pharmacokinetics investigation demonstrates that the nUK has the maximum blood concentration in the animals after sonication, whereas before that, it kept at a low level close to that of the control group (Fig. 2). Since the ultrasound wave can be focused at the stroke site, it infers that the release of free uPA is around the clot area where it should be at the highest concentration and thus would favor the thrombolysis action of the protein. Besides the triggered release, the US treatment in the nUK+US group also applied an acoustic radiation energy to loosen the thrombus via localized mechanical stress and thus facilitate the nUK and/or uPA penetration, which eventually led to the best therapeutic efficiency among treated animals (Figs. 4 and 5). Comparably, sonication to the animals dosed by free uPA, i.e. the UK+US group, showed limit improvement in the thrombolysis when compared to that without the ultrasonication (Figs. 4 and 5), which can be attributed to the non-specific delivery and the fast clearance of the uPA (Fig. 2).

It is known that efficient thrombolysis treatment is frequently accompanied by high risk of HT because the circulation of free uPA can cause the BBB destruction after the ischemic stroke [60,61]. In this work, we show that in the nUK+US group, the BBB impairment

was even reduced as demonstrated by the EB concentration (Fig. 6a), possibly due to the relatively small infarct volume caused by the localized release of the protein. Besides, the use of diagnostic ultrasound, i.e. 2 MHz, for the intervention may be also taken in concern. As a result, the administration of the uPA loaded nanogels harvested no damageable results on the safety evaluation such the concentration of Hb, the HT and death rates. The overall data thus support that the nanogel formation is promising in the sonothrombolysis treatment of stroke. Nevertheless, the efficiency of the sonothrombolytic system on treating larger feeding carotid clots is to be further investigated.

#### 4. Conclusions

In summary, in this work we built rat model of suture embolization-induced acute ischemic stroke for assess the capacity of ultrasound sensitive urokinase-loaded nanogels (nUK) on the amelioration of the stroke severity. Results exhibit that the gas-free nanogels could significantly increase the effectiveness of thrombolysis upon ultrasonic intervention when compared to the dose of free uPA which has shown no statistical difference between the thrombolysis and sonothrombolysis treatments. And this effect was accompanied by the well-protection of the BBB integrity without adverse brain hemorrhage and animal death after 1 week of the nUK administration. Therefore, our work provides new insights into the sonothrombolysis treatment of ischemic stroke by the use of new ultrasonic sensitive carrier of uPA.

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