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Original Article

Preparation of arginine-glycine-aspartic acid (RGDS)-Urokinase-carrying targeting ultrasound contrast agent by avidin-biotin system and its impacts on thrombus-targeting affinity

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Abstract: *Objective:* This study aimed to prepare the arginine-glycine-aspartic acid (RGDS)-urokinase-carrying targeting ultrasound contrast agent using the avidin-biotin system (BAS) and to investigate its physicochemical properties and thrombus-targeting affinity. *Methods:* The ultrasound contrast agent (Targestar SA) was firstly combined with different doses of RGDS and urokinase by BAS, the products were then tested the binding rate and stability by flow cytometry. The rabbit femoral arterial thrombus model was then used to study the targeting affinity of this ultrasound contrast agent towards thrombus. *Results:* The results of flow cytometry revealed that the Targestar SA microbubbles successfully carried different doses (3 mg, 6 mg) of RGDS and urokinase (RU-SA), the binding rate was more than 98%, and the stability was not affected by the rinsing. *Conclusions:* Fluorescence microscopy revealed that RU-SA had targeting affinity towards thrombus. RU-SA prepared by BAS had high carrying rate and exhibited targeting affinity towards thrombus *in vivo*.

Keywords: Contrast agent, microbubbles, urokinase, thrombolysis

Introduction

Targeting thrombolysis microbubble contrast agent has special affinity towards thrombus tissues, so it could improve the diagnostic specificity towards thrombus, play the role of targeting thrombolysis, and could be expected to rapidly improve the clinical safety and efficacy of current thrombolytic therapies [1]. Existing studies [2-6] mostly combined the microbubble with certain drug or with certain targeting ligand; however, whether these three substances could adequately and stably bind with each other, thus playing the roles of targeting thrombus, has been less reported.

Studies had confirmed that [7, 8] the short peptide containing the gene sequence of arginine-glycine-aspartic acid (RGDS) could be used as one thrombus targeting ligand and could specifically bind with the glycoprotein GPIIb/IIIa receptor on the activated platelet surface, so these microbubbles could specifically and firmly combine with the activated platelets in thrombus, thereby assisting the targeting thrombolytic therapy.

Experimental studies had confirmed the feasibility of adhering the ligand-carrying microbubbles to the targeting bodies under certain conditions [9, 10]. Avidin-biotin system (BAS) has become one commonly used method in preparing ultrasound contrast agents [11, 12]. In this study, new contrast agent Targestar SA, with streptavidin conjugated onto its surface, was used as the targeting microbubble contrast agent, the biotin-labeled ligand (RGDS and urokinase) were then linked onto the microbubble surface via the chemical integration of BAS; RGDS then could specifically bind with the surface target of thrombus tissues and targetedly play the thrombolytic roles of urokinase.

Focusing on whether the microbubble contrast agent, targeting ligand, and drug could be bound sufficiently and stably, this study preliminarily investigated the feasibility and methodology of preparing this targeting microbubble con-

Table 1. pH measurement results of different groups

Group	Ingredients	Conditions	pH
1	200 ul of Targestar SA	--	6.5
2	200 ul of Targestar SA + 3 mg of FITC-UK + 3 mg of 5-TAMRA-RGDS	Non-rinsing	4.8
3	200 ul of Targestar SA + 3 mg of FITC-UK + 3 mg of 5-TAMRA-RGDS	Rinsing	6.3
4	200 ul of Targestar SA + 6 mg of FITC-UK + 6 mg of 5-TAMRA-RGDS	Non-rinsing	4.4
5	200 ul of Targestar SA + 6 mg of FITC-UK + 6 mg of 5-TAMRA-RGDS	Rinsing	6.1

trast agent; furthermore, referring to the common dose of urokinase used in rabbit femoral arterial thrombus model [13], the carrying rates of different doses of urokinase by this microbubble was detected to further confirm the stability among these three substances. Moreover, the prepared targeting contrast agent was then intravenously injected into the animal thrombus model to study its thrombus-targeting affinity in vivo.

Materials and methods

Preparation of RGDS and Urokinase (RU-SA)

Urokinase (Batch number 20071011, raw material of injection-grade, Nanjing Nanda Biochem Co. Ltd., Jiangsu) was firstly performed the fluorescein isothiocyanate (FITC) labeling, and the product would display bright yellow-green fluorescence. According to the instructions of Thermo and the ratio of protein: Biotin (or FITC)=1:15, 4.17 mg of Biotin and 3.55 mg of FITC were weighed and dissolved in pure water (Biotin) or DMSO (FITC), respectively; the dissolved Biotin and FITC were then simultaneously added into the urokinase solution and reacted for 1 h in darkness; after the reaction, the above solution was dialyzed with PBS (pH 7.4) overnight at 4°C, followed by another consecutive 5 h dialysis after changing the medium on the next day; the product was then harvested and stored in darkness at -20°C.

The RGDS peptide was performed the 5-carboxyltetramethylrhodamine (5-TAMRA) labeling, and the product would display orange-red fluorescence, which would exhibit significant contrast to the yellow-green fluorescence of FITC. According to the instructions of Thermo, Biotin and Rhodamine (1:1, w/w) were weighed and dissolved in pure water (Biotin) or DMSO (Rhodamine), respectively, with the final concentrations both as 10 mg/mL; the dissolved Biotin and Rhodamine were then simultaneously added into the RGDS solution and reacted for 1 h in darkness; the product was then harvested and stored in darkness at -20°C.

According to the ratio of urokinase/RGDS and rinsing conditions, this study was divided into five groups (**Table 1**). Using one one-ten-thousandth electronic balance, a certain amount of fluorescence-labeled urokinase (FITC-UK) and RGDS (5-TAMRA-RGDS) was accurately weighed firstly; meanwhile, according to the instructions of ultrasound contrast agent, Targestar-SA (Country of Origin: USA, provided by Nanjing Yuanduan BioTech Co., LTD) was gently shaken until a white milky suspension of microbubbles was obtained. 200 μl of this microbubble suspension was then mixed with different doses of FITC-UK and 5-TAMRA-RGDS, followed by the incubation and standing at room temperature. The specific groupings were shown as follows.

After the above-described preparation process, the mixture was incubated at room temperature for 30 min; then, one half of the mixture was taken for still standing, and the other half was rinsed twice using the flotation method [14]. The prepared specimens were then detected the physicochemical properties.

Appearance of the contrast agent

The products of different groups were stood still for certain period, their appearance was visually observed.

Detection of average particle diameter, concentration, and pH of the microbubbles

The average particle size and concentration of the targeting microbubbles were measured using one Coulter counter, and a small amount of microbubble suspension was sampled from each group for the pH detection using a precision pH meter.

Optical microscopy and fluorescence microscopy

A small amount of microbubble suspension was sampled from each group and dropped on a

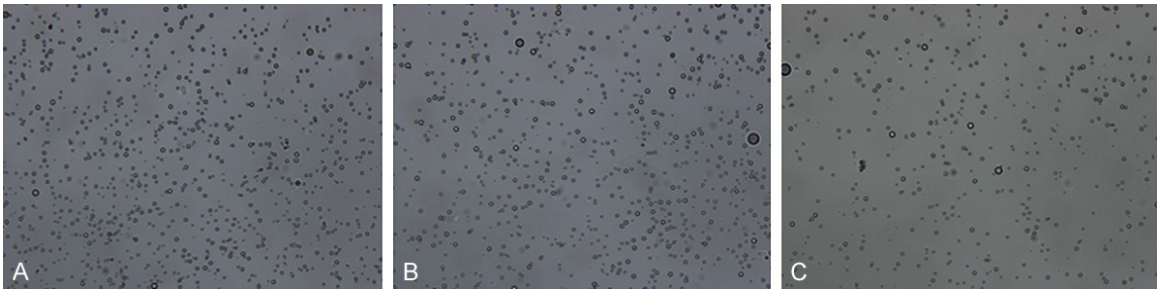

Figure 1. A. Optical microscope observation of Targestar SA microbubble contrast agent (×200); B. Optical microscope observation of Targestar SA microbubble contrast agent + 3 mg of urokinase + 3 mg of RGDS (×200); C. T Optical microscope observation of Targestar SA microbubble contrast agent + 6 mg of urokinase + 6 mg of RGDS (×200).

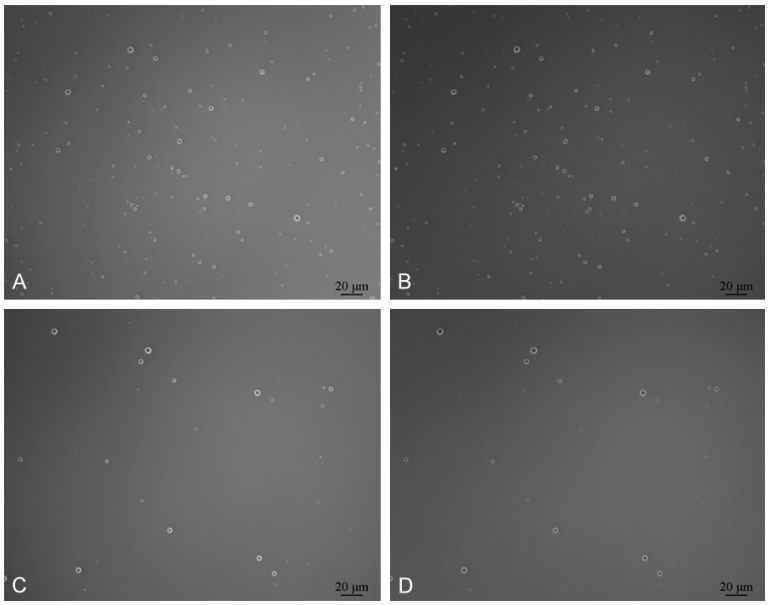

Figure 2. Fluorescent microscopic observation of Targestar SA + 3 mg of urokinase + 3 mg of RGDS microbubble contrast agent (×200). The above figures were the results of the same sample using two different excitation spectra (490 nm and 546 nm): A (before rinsing) and C (rinsed): the microbubble wall exhibited circular green fluorescence; B (before rinsing) and D (rinsed): the microbubble wall exhibited circular orange-red fluorescence.

glass slide to observe the form and size of the microbubbles before and after rinsing using an optical microscope. In addition, microbubble suspension was observed the fluorescence-labeling situations of urokinase and RGDS under different excitation spectra of one fluorescence microscope (LEICA CTR6000, Germany).

Flow cytometry

Flow cytometry (Beckman, USA) was performed to analyze the binding rates of urokinase and RGDS and the possible related factors.

Stability detection of different RU-SA microbubbles in rabbit femoral arterial thrombosis model

The rabbit femoral arterial thrombus model was prepared referring to the previous study [15]; different RU-SA suspensions (FITC-UK and 5-TAMRA-RGDS, respectively) were then injected into rabbits along the ear vein. After confirming the microbubbles had aggregated onto the thrombus, the rabbit was sacrificed with excessive anesthetic, and the femoral arterial specimens were sampled to observe whether there existed the expression of fluorescent substance on the in vitro thrombus surface using a fluorescence microscope.

Results

Appearance and optical microscopic observations of RU-SA

After mixed evenly, Targestar SA exhibited one milky white liquid, and after standing still, this liquid might easily stratify with the upper layer as white microbubbles and the lower layer as clear and transparent liquid. The appearance of different groups under an optical microscope was shown in **Figure 1**: the microscopic morphologies of these three groups did not very significantly, the microbubbles were smooth, small, and spherical, the annular outer shell

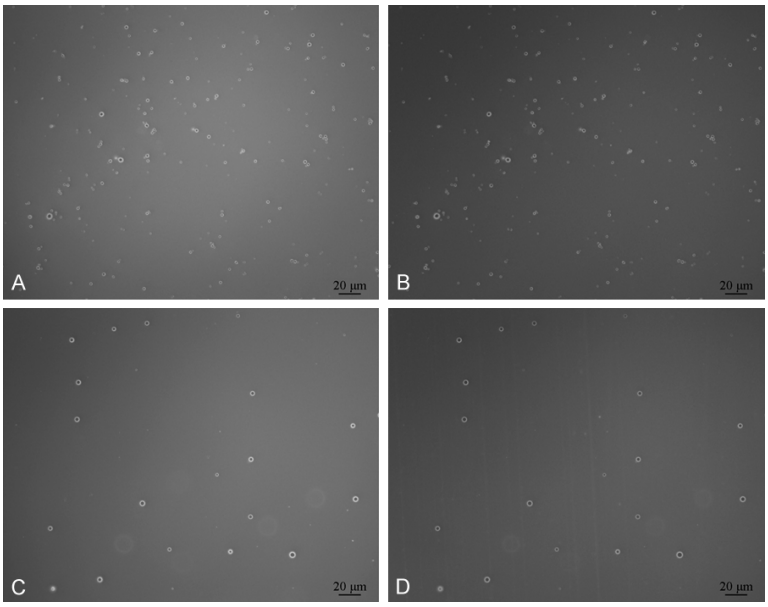

Figure 3. Fluorescent microscopic observation of Targestar SA + 6 mg of urokinase + 6 mg of RGDS microbubble contrast agent (×200). The above figures were the results of the same sample using two different excitation spectra (490 nm and 546 nm): A (Before rinsing) and C (Rinsed): the microbubble wall exhibited circular green fluorescence; B (Before rinsing) and D (Rinsed): the microbubble wall exhibited circular orange-red fluorescence (×200).

surrounded the central translucent area, the sizes were relatively uniform, and no aggregation appeared. The average particle diameters and concentrations of group A, B, and C obtained by the Coulter Counter were 2.03±1.08 μm and 3.55×10^8 cells/ml, 2.45±1.03 μm and 3.98×10^8 cells/ml, and 2.43±1.19 μm and 3.79×10^8 cells/ml, respectively. The pH measurement results of different groups were shown in **Table 1**.

Fluorescence microscopic observation

Under a fluorescence microscope, each sample was observed after excited by two different excitation spectra (490 nm and 546 nm) (**Figures 2** and **3**).

It could be seen by fluorescence microscopy that the surface of the microbubbles that combined with different doses (3 mg and 6 mg) of urokinase and RGDS exhibited the same green fluorescence as FITC-UK and the same orange-red fluorescence as 5-TAMRA-RGDS, the staining of the microbubble shell was uniform and circular. After rinsing, the microbubbles showed no significant change, namely before and after rinsing, the microbubbles were annular with their surface still showing green and orange-red fluorescence, and the fluorescence brightness showed no significant change.

Flow cytometry

The binding rates of different groups were detected using flow cytometry, and the results were shown in **Figure 4**.

The above results indicated that the binding rates of all group were more than 98%, indicating no significant difference. Different doses of urokinase + RGDS (3 mg and 6 mg) had no impact on the carrying rate of the microbubbles, and rinsing or not had no impact on the binding rate of urokinase and RGDS.

Stability detection of RU-SA in rabbit femoral arterial thrombus model

The prepared suspension of RU-SA ultrasound contrast agent (fluorescence-labeled) was injected through the rabbit ear vein, and the Mylab90 color Doppler ultrasonic diagnostic apparatus (ESAOTE, Italian) equipped with LA523 probe (frequency 4-13 MHZ) was used to real-time monitor and observe the femoral artery using the contrast mode and 2D model. Under the ultrasonic contrast mode, it could be seen that the contrast agent filled the target femoral artery and aggregated at the thrombus (**Figure 5**).

The rabbit was then sacrificed with excessive anesthetic, and the femoral arterial thrombus specimen was then sampled for the observation using a fluorescence microscope. As shown in **Figure 6**, when the excitation wavelength was 490 nm, the surface of the rabbit in vitro thrombus displayed green fluorescence; when the excitation wavelength was 546 nm, the orange-red fluorescence could be clearly seen, thus confirming that the FITC-UK and 5-TAMRA-RGDS microbubbles all adhered to the surface of the thrombus.

Discussion

It has been shown in certain study that the combination of targeting microbubbles, ultra-

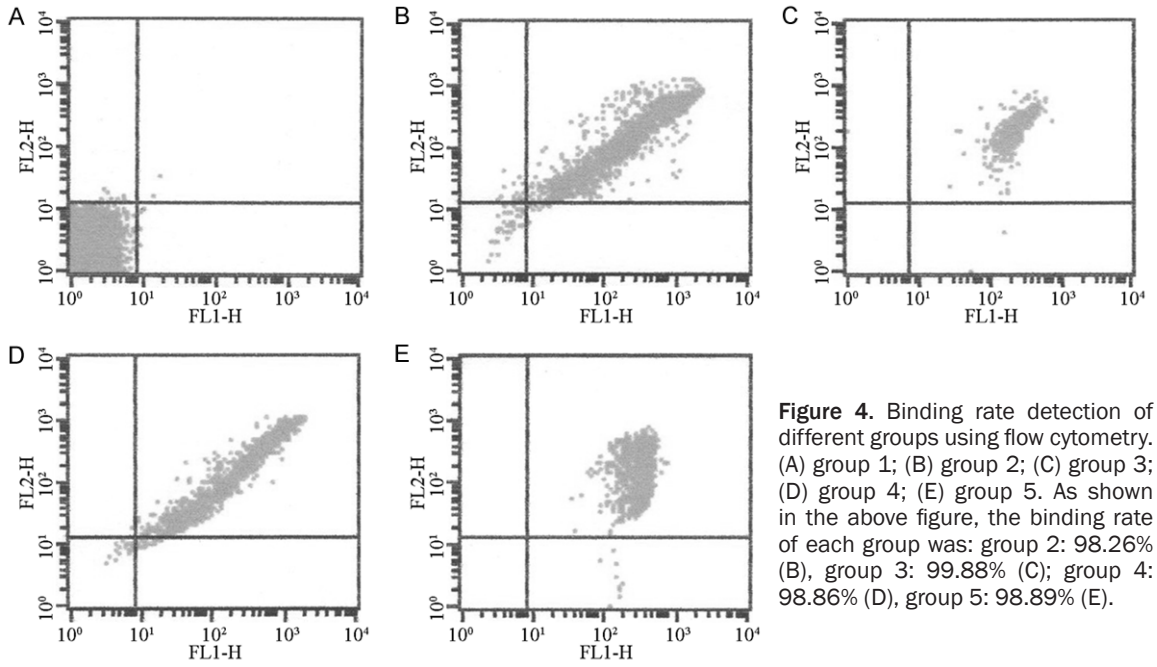

Figure 4. Binding rate detection of different groups using flow cytometry. (A) group 1; (B) group 2; (C) group 3; (D) group 4; (E) group 5. As shown in the above figure, the binding rate of each group was: group 2: 98.26% (B), group 3: 99.88% (C); group 4: 98.86% (D), group 5: 98.89% (E).

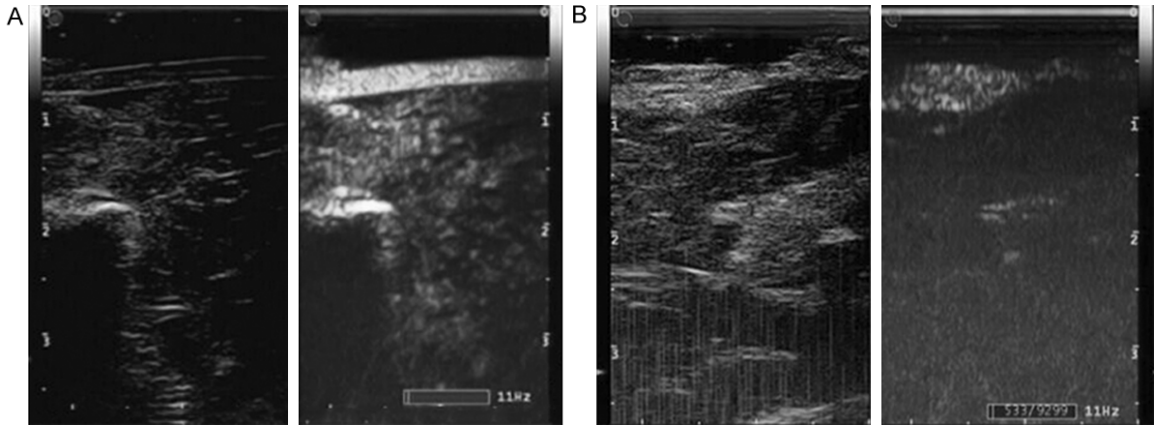

Figure 5. Under the ultrasound contrast mode, it could be seen that before forming the bolt, the contrast agent filled the target femoral artery (A); after the thrombus formed, the contrast agent filled the target femoral artery and aggregated at the thrombus (B).

sound, and thrombolytic drug could exhibit the best thrombolysis effects; meanwhile, it could reduce the possible side effects when using the drug or ultrasonic thrombolytic therapy alone [16-18]. Many researches have focused on the combination of microbubbles with drug or gene, as well as the combination of microbubbles with targeting ligands [2-6, 19], but it was rarely reported about whether the microbubble contrast agent, targeting ligand, and drug could sufficiently and stably bind together and then play the targeting thrombolysis effects. In this study, after the microbubbles carried UK and RGDS, the light microscopy revealed that the microbubbles were of relatively uniform size, did not aggregate, and exhibited the particle size still smaller than that of red blood cells, consistent with the characteristic requirements of targeting microbubble contrast agent [1]. This study rinsed the targeting microbubble contrast agent, but regardless of rinsing, the microbubbles kept their annular outline. The post-rinsing binding rate was not reduced, suggesting that the combination of the ligand with the microbubble was stable. Furthermore, after rinsing, the pH values of all groups were above 6.0, which was more in line with the physiological characteristics of the

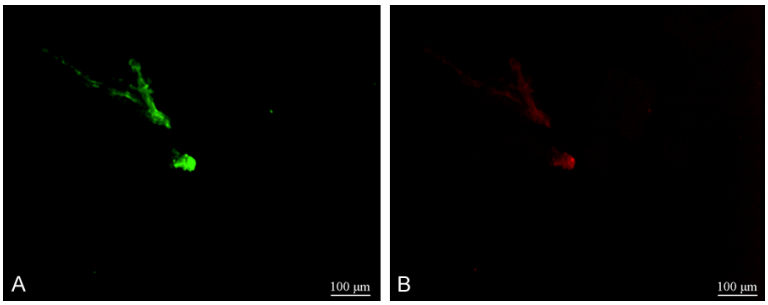

Figure 6. Conditions of green FITC-UK (A) and orange-red 5-TAMRA-RGDS (B) on the surface of the rabbit in vitro thrombus.

internal environment and would not interfere the in vivo acid-base balance too much.

How to further improve the binding stability between the targeting microbubbles and drug or ligand is a concern in clinical thrombolytic studies. Recently, BAS is commonly used, and it's one of the most effective ways to connect the ligand onto the microbubbles currently [11, 12]. As a targeting microbubble contrast agent, Targestar SA has been used in a number of animal studies. In studying tumor angiogenesis, Wei *et al.* [11] applied Targestar SA, conjugating streptavidin and angiogenesis-molecular marker (anti-VEGFR antibody) on its surface, as a targeting ligand to reach the targeting site (where the renal cell carcinoma located); compared to other normal contrasts, this new contrast agent enhanced the imaging of the angiogenetic site and had a longer and enhanced residence times. Another study [14] using the murine colon cancer model applied Targestar SA to carry anti-angiogenic drugs, and then used targeting ultrasonic cavitation to realize the targeting anti-tumor therapy. In this study, FITC-UK and 5-TAMRA-RGDS were chemically linked onto the surface of Targestar SA by BAS, expecting that RGDS could act as a ligand and specifically bind with the surface site on the thrombus; so that the microbubbles could stay longer at the thrombus and targetedly play the thrombolytic role of urokinase.

The doses of urokinase designed in this study included conventional dose (3 mg) and doubling dose (6 mg), aiming to detect the binding rate of the microbubbles when combined with different doses of urokinase. The results proved the Targestar SA microbubbles successfully carried different doses of urokinase and RGDS by BAS. The fluorescence microscopic observation revealed that the surface of the microbubbles carrying different doses (3 mg and 6 mg) of urokinase and RGDS displayed the same green fluorescence as FITC-UK and the same orange-red fluorescence as 5-TAMRA-RGDS; meanwhile, the shell of the microbubbles was stained evenly and showed annular form. The results showed that urokinase and RGDS were successfully connected to Targestar SA. The green and orange-red fluorescence both exhibited the "annular form", indicating that urokinase and RGDS were located on the surface of the microbubbles. Certain study reported flow cytometry as an effective method for the quantitative detection of microbubbles. The results of flow cytometry in this study showed that the binding rates of different doses (3 mg and 6 mg) of urokinase and RGDS on the microbubbles were all more than 98%, suggesting that BAS was one of the most effective ways currently to connect the ligand with the microbubbles and would not be influenced by the doses of RGDS and urokinase. In our previous studies, flow cytometry showed that the binding rate of urokinase and RGDS on the shell of SonoVue microbubbles by the direct connection method was less than 80% [7].

In recent years, scholars combined GPIIb/IIIa receptor ligand (RGDS) with microbubble contrast agent during their thrombolytic experimental studies [7, 8], and the targeting of this prepared targeting microbubble contrast agent was tested; the results showed that the platelet GPIIb/IIIa receptor targeting microbubble contrast agent obviously adhered onto the thrombus *in vitro* and *in vivo* and could enhance the ultrasonic identification signal of the thrombosis, which exhibited great clinical significance for the clinical diagnosis and treatment of thrombus. In this study, fluorescence microscopy revealed that the fluorescent substances could be seen on the in vitro thrombus, thus confirming that RU-SA adhered onto the surface of the thrombus. These results suggested that RU-SA had stable and thrombus-targeting thrombolytic effects *in vivo*.

In summary, this study successfully used BAS to prepare targeting ultrasound contrast agent, and different doses of urokinase and RGDS were combined onto the surface of the micro-

bubbles; this targeting contrast agent was then intravenously injected into the animal thrombus model, and it's stability and targeting were confirmed. The in vivo animal experiments have been focused on the targeting thrombolytic efficacies and optimal ultrasound parameters [20, 21], thus providing theoretical and experimental evidence for the clinical therapy of targeting microbubble contrast agent plus thrombolytic drugs.

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Disclosure of conflict of interest

None.

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