Dual-Modality Imaging for Monitoring Thrombosis via Platelet GPIIb/IIIa Receptor Targeted cyclic RGDfK Microbubbles

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Abstract

**Background:** Acute thrombotic events play a vital role in cardiovascular diseases. Our study aims to investigate an ultrasound microbubble for dual-modality imaging of thrombi.

**Methods:** DID-DSPE-PEG-cRGD-Microbubbles were prepared by mechanical vibration and Chemical chelation methods. *In vivo* fluorescence and ultrasound imaging were performed after intravenous injection of DID-DSPE-PEG-cRGD-Microbubbles respectively.

**Results:** This microbubbles exhibited good binding affinity to activated platelets with significant fluorescence and ultrasonic signals. Ultrasound and fluorescence imaging revealed that the microbubbles accumulated at the site of the thrombus in the carotid artery, and immunofluorescence showed that microbubbles could be detected in the carotid artery thrombus.

**Conclusion:** We successfully synthesized novel microbubble cRGD-MBs, which indicate excellent potential for immediate diagnosis of acute thrombus *in vivo.*

Introduction

Cardiovascular disease is a major cause of mortality and morbidity worldwide.[1] Thrombosis is an important pathophysiological phenomenon in various cardiovascular diseases, leading to tissue hypoxia and infarction.[2] Typically, the rupture of unstable atherosclerotic plaques results in endothelial injury followed by platelet adhesion to the extracellular matrix, platelet activation, and finally the release of various cytokines. This ultimately leads to thrombosis and vascular occlusion, causing hypoxia and tissue necrosis, which requires timely diagnosis and immediate management.

Many imaging techniques have been developed to visualize fibrin and platelet reactions.[3] However, noninvasive fast detection of thrombus remains a challenge. Multi-modality imaging based on nanoparticles has provided opportunities for detecting biological changes of thrombus *in vivo* with activatable nanoprobe. We have constructed Osteopontin-targeted (OPN-targeted) magnetic resonance/optical dual-modality molecular probes to identify vulnerable plaques in atherosclerotic models.[4, 5] Arterial thrombosis caused by rupture of unstable atherosclerotic plaques occurs suddenly and progresses rapidly. Among all imaging modalities, ultrasound imaging has several advantages of non-invasive, no radiation-associated risk and no side effects, which are highly portable and available in most hospitals. Therefore, molecular ultrasound imaging could provide a safe, rapid, and cost-effective technique for detection of thrombosis. Targeted ultrasound microbubbles can significantly enhance the signal of the target lesion by accumulated microbubbles with surface ligands binding to corresponding receptors of thrombus, which has a good prospect in real-time, rapid, and accurate diagnosis of thrombosis.[6, 7]

Platelets play a pivotal role in thrombosis, and the GPIIb/IIIa receptor is an eventual pathway of platelet activation.[8] Once platelets are activated, GPIIb/IIIa undergoes a conformational change from a low-
affinity to a high-affinity state, allowing the binding of fibrinogen, which leads to platelet aggregation and thrombus formation.\textsuperscript{[9,10]} The change in GPIIb/IIIa conformation could distinguish activated from non-activated platelets, making it an ideal biomarker target for monitoring thrombosis. Binding of soluble fibrinogen to GPIIb/IIIa in activated platelets is mediated exclusively by the C-terminal AGDV-containing dodecapeptide (γC-12) sequence of the fibrinogen γ chain.\textsuperscript{[11,12]} Previous studies revealed that peptides containing the Arg-Gly-Asp (RGD) sequences located in the fibrinogen Aα chain can make substantial contributions to GPIIb/IIIa binding when fibrinogen is immobilized and converted to fibrin. Furthermore, cyclic RGD peptides (cRGDfK) have shown a higher binding affinity to GPIIb/IIIa than linear peptides.\textsuperscript{[13,14]} Recent studies also revealed that cRGDfK and AGDV-containing peptides are potent inhibitors of the GPIIb/IIIa fibrinogen interactions, and cRGDfK has a stronger affinity,\textsuperscript{[14]} which makes cRGDfK a potential ligand to recognize activated platelets.

Herein, we have developed a novel ultrasound/fluorescence dual-imaging microbubble via phospholipid microbubbles containing cRGDfK peptide (denoted as cRGD-MB), which can rapidly bind to the GPIIb/IIIa receptor of activated platelets and accurately locate fresh thrombi for ultrasound/fluorescence dual-modality imaging.

**Materials and Methods**

**Animal modeling of Carotid thrombosis**

All animal studies and procedures were performed according to the protocol approved by the Chinese PLA General Hospital Animal Care and Use Committee in accordance with the Care and Use of Laboratory Animals formulated by the National Society for Medical Research. The approval number of the laboratory was S2020-285-01. 8-week-old male C57/BL6 mice weighing 15–20 g were purchased from Vital River, Beijing.

**Materials**

The compound DSPE-PEG-cRGD and DSPE-PEG was purchased from ChinaPeptides (Shanghai, China). DSPC (1,2-distearoyl-\textit{sn}-\textit{glycero}-3-phosphocholine), DiD (1,1-dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine perchlorate), and perfluoropropane (\textit{C}_3\textit{F}_8) gas was provided by the College of Engineering of Peking University. Tirofiban and adenosine diphosphate (ADP) were provided by the Chinese PLA General Hospital. FITC-labeled CD62p antibody was purchased from Biorbyt (Wuhan, China). Rabbit polyclonal CD62p antibody and FITC-conjugated goat anti-rabbit IgG antibody were purchased from BD Biosciences. Propanediol, glycerine, and PBS (pH = 7.4) were purchased from Solarbio (Beijing, China). All materials were used without any further purification. In this study, the synthesis of DSPE-PEG-cRGD was performed using ChinaPeptides (Shanghai, China).

**Preparation of cRGD-MBs:** A microbubble composed of DSPC and DSPE-PEG-cRGD was prepared by the film-sonication method, non-targeting microbubble composed of DSPC and DSPE-PEG was prepared for
control. Initially, DSPC and DSPE-PEG-cRGD were dissolved in ethanol to obtain a solution with a concentration of 1 µmol/mL and 0.9 mL of DSPC (1 µmol/mL), 0.1 mL DSPE-PEG-cRGD (1 µmol/mL), 2 µL DID (dissolved in ethanol, 1 µmol/mL), and 0.1 mL propanediol were mixed in 2 mL Eppendorf tubes, followed by drying in a termovap sample concentrator at 60°C for approximately 20 min to form propylene glycol solution of lipids. A mixture of glycerol and phosphate buffered saline (PBS) was prepared in a volume ratio of 1:10, and then 1 mL of a mixture of glycerol and PBS was added to the resulting solution at 55°C and hydrated for 30 min in a water bath ultrasonic environment to obtain the liposome. The liposomes were collected and put in penicillin bottles. Next, C₃F₈ was introduced into the vial for 1 min to place the air and was sealed carefully. Finally, a vialmix mechanical agitator was employed to generate microbubbles by violent vibration for 45s, the shaking frequency of the mechanical agitator was 4530 ± 100 oscillations per minute. the microbubbles were stored at 4°C until use.

**Characterization of cRGD-MBs**

Prepared cRGD-liposome were diluted to 1/500, and the sample was dripped on the copper net containing carbon support film. After 3 minutes, the excess liquid was absorbed by the filter paper from the edge, and 3% phosphotungstic acid solution was added. After dyeing for 2 minutes, the excess dye solution was absorbed by filter paper, then washed with distilled water for 3 times, then observed by transmission electron microscope after the copper mesh was dried. Morphology analysis of cRGD-liposomes was carried out by TEM (HT-7700). Fluorescent images of microbubbles were acquired using a fluorescence microscopy (Leica DMI3000B). The size distribution and concentration of microbubbles was measured with a Coulter Counter (Multisizer 3 Coulter Counter, Beckman Coulter, Inc., USA.), in which 20µL samples were diluted in 20mL electrolyte solution, and the orifice diameter used was 20µm, the analysis range was 0.4–12.0µm. Fluorescence spectra were recorded using a fluorescence spectrophotometer (Lumina, Thermo Scientific). *In vitro* ultrasound imaging of cRGD-MBs was performed using *in vitro* ultrasound imaging in a latex tube using a clinical US imaging system (Mindray resona 7 ultrasound system) in B-mode and contrast mode with various concentrations of cRGD-MBs (0.1, 0.5, and 1.0x10⁷ MBs/mL), grayscale values of ultrasound images were calculated using Image J software for semi-quantitative analysis.

*Specific binding ability of cRGD-MBs on platelets*: Blood from healthy adult volunteers was collected in sodium citrate 3.8% (w/v). Platelet-rich plasma (PRP) was obtained by centrifugation at 180g for 10 min and diluted to 1:10 in PBS. Activated platelets were obtained via stimulation with adenosine diphosphate (ADP, 20 µM). GPIIb/IIa blocked platelets were obtained by incubation with tirofiban at a high concentration (50 µg/mL). Before assessing the affinity between the cRGD-MBs and activated platelets, PRP, PRP + ADP, and PRP + ADP + tirofiban were prepared and incubated with FITC-labeled CD62p antibody to evaluate platelet activation. Then, the tubes with PRP, PRP + ADP, and PRP + ADP + tirofiban were incubated with cRGD-MBs at certain concentrations for 20 min. After washing three times with PBS, samples were analyzed by flow cytometry with 10,000 events collected per sample. The mean fluorescence intensity was tested for the quantitative analysis of the affinity between the cRGD-MBs and activated platelets.
To measure the binding ability of cRGD-MBs to activated platelets, platelets were stimulated with ADP (20µM), incubated with FITC-labeled CD62p antibody, and cRGD-MBs for 30 min at RT. After washing three times with PBS, the droplet was placed on the slide, and the cover glass moved evenly from left to right at an angle of 35–40 degrees after contacting the drop, leaving a layer of blood film, finally, sealed with glycerin. Photographs were taken using a laser scanning confocal microscope.

*In vivo thrombus imaging*: A carotid thrombosis model was established in mice. For *in vivo* thrombus imaging, C57/BL6 mice with a body weight range of 15–20 g were selected and anesthesia was induced by isoflurane at a concentration of 4% and maintained at a concentration of 2% with oxygen as the carrier. Left common carotid artery (CCA) thrombosis was formed by covering the exposed carotid artery with filter paper infiltrated by 10% FeCl₃ solution for 3 min. For *in vivo* fluorescence imaging, mice were divided into four groups (five mice in each group): sham operation, thrombus + cRGD-MBs, thrombus + non-targeting MBs, and tirofiban pretreated group. A suspension of 100 µL microbubbles was injected via the tail vein into each group. *In vivo* fluorescence images of the thrombus were acquired using the IVIS Spectrum imaging system (PerkinElmer, USA). In order to confirm whether the fluorescence signal was accurately located at the thrombus site, the carotid artery in the thrombus group and tirofiban pretreated group was dissected from the mouse 120 min post-injection of cRGD-MBs and separate imaging. To determine the metabolic pathways of cRGD-MBs, five mice were sampled 120 minutes after injection of cRGD-MBs, fluorescence imaging of organs was performed and the intensity of each organ was recorded, and the mean values of fluorescence intensity of different organs were calculated to evaluate the metabolism of microbubble *in vivo*. Thus, the analysis of the fluorescence intensity of different groups was also completed by the image analysis software carried by the imaging system. For ultrasonic imaging, the experimental animals were divided into four groups (five mice in each group): thrombus + cRGD-MBs, thrombus + non-targeting MBs, thrombus + cRGD-MBs + tirofiban and thrombus + no microbubble. After continuous observation by ultrasound for 10 min, B-mode images and contrast mode images of each experimental animal were recorded, both before and after surgery. The carotids were imaged using an ultrasound system with a high-frequency probe (40 MHz) in the long-axis view.

*Histological analysis of the in situ thrombotic model*: For histological analysis, the left CCA of the carotid artery thrombus group and the tirofiban pretreated group was isolated after perfusion with 4% paraformaldehyde via the left ventricle and embedded in paraffin. The sliced CCAs (5 µm) were stained with hematoxylin and eosin (H&E) and observed by optical microscopy. Fluorescent signals of both groups were observed after immunofluorescence staining at the thrombus site. For thrombus staining, the CCA tissue slide was washed twice with PBS (pH 7.4) and incubated with blocking solution for 1 h at RT. Then, the CCA tissue slide was washed twice with PBS and incubated with rabbit anti-mouse polyclonal CD62p antibody (BD biosciences) (0.15 mg/mL, 0.3% BSA containing PBS [pH 7.4]) for 2 h at RT, and the CCA tissue slide was then washed twice with PBS. For visualization of thrombin, FITC-conjugated goat anti-rabbit IgG antibody (BD biosciences) (1:1,000, 0.3% BSA containing PBS [pH 7.4]) was added to the CCA tissue slide for 1 h at RT. Finally, the CCA tissue slide was washed twice with PBS and mounted.
using a cover glass. The fluorescence in the CCA tissue was observed using a laser scanning confocal microscope.

**In vivo toxicological analysis**

For the *in vivo* toxicological analysis of cRGD-MBs, major organs (liver, lung, spleen, kidney, and heart) were dissected from mice 90 min after *i.v.* injection of PBS, cRGD-MBs (10 mg/kg), and cRGD-MBs + tiroban, respectively. Then, tissues were harvested and fixed with 4% paraformaldehyde solution and embedded in paraffin. The sliced organs (6 µm) were stained with H&E and observed using an optical microscope.

**Statistical analysis**

All statistical tests were performed using the Statistical Package for the Social Sciences software version 19.0 (SPSS Inc., Chicago, IL, USA). Values are expressed as the mean ± standard deviation (M ± SD) of five replicates in each experiment. Statistical differences between groups were determined by one-way analysis of variance (ANOVA) followed by the Student's unpaired t-test. A *p* value < 0.05 was considered statistically significant.

**Results**

**Synthesis and characterization of cRGD-MBs**

We constructed ultrasound/fluorescent dual-modality microbubbles using the thin-film dispersion method (Fig. 1A). TEM images demonstrated that the diameters of cRGD-liposomes ranged from 200 nm to 400 nm (Fig. 1B). The cRGD-MBs were well dispersed, and emitted bright red fluorescence under the excitation wavelength of 660 nm (Fig. 1C). The fluorescence spectrum (Fig. 1D) of cRGD-MBs indicated that DiD fluorescent dyes were uniformly distributed on the surface of the microbubbles. The diameter of cRGD-MBs was approximately 1–3 µm, as measured by a coulter cell counter (Fig. 1E).

**In vitro specific binding to activated platelets of cRGD-MBs**

The ability of cRGD-MBs to target platelets GPIIb/IIIa was evaluated *in vitro* using platelet-rich plasma, and the activation marker CD62p was used as a positive control. The affinity of cRGD-MBs to resting platelets, activation platelets, and platelets pretreated with tiroban was evaluated by flow cytometry, and platelet activation in each group was pre-determined by FITC labeled CD62p. In vitro fluorescence imaging showed that cRGD-MBs exhibited more strong fluorescence signal intensity when higher affinity in ADP (adenosine diphosphate)-activated platelets than in either resting platelets or platelets pretreated with GPIIb/IIIa inhibitors (GPI) tiroban (*P* < 0.05)(Fig. 2A and 2B). We also measured the affinity of cRGD-MBs to resting and activated platelets under a confocal microscope. *In vitro* fluorescence imaging showed that the resting platelets were dispersive of no obvious fluorescence signal. However, the activated platelets (PRP + ADP) were reunion and exhibited significant green fluorescence signal from the
FITC-labeled CD62p antibody and red fluorescence signal of cRGD-MBs. The merge of fluorescence signals revealed that the distribution of cRGD-MBs was consistent with activated platelets. (Fig. 2C). Semi-quantitative analysis of the imaging revealed that, cRGD-MBs showed higher fluorescence intensity than CD62p-FITC after binding to activated platelets (Fig. 2D).

**Ultrasound imaging of in situ thrombotic animal model**

To observe the effect of cRGD-MBs on the ultrasound imaging of thrombus, we firstly tested the imaging ability of microbubbles *in vitro* (Fig. 3A). In the latex tube imaging test of microbubbles, semiquantitative analysis results indicated that the imaging signal intensity enhanced with the increase of microbubble concentration (Fig. 3B). In order to observe the ultrasound imaging effect of cRGD-MBs with thrombus, we observed the entire process from the beginning of modeling to the formation of a thrombus after the administration of microbubbles in three modes. Before the thrombosis model, the carotid artery of mice was observed in the long-axis view, and images of B-mode and contrast mode were collected. Carotid artery had a clear boundary. Under invariable conditions, maintain the position and angle of the complete thrombosis model. After injecting cRGD-MBs intravenously via the tail vein, signal of microbubble filling the whole blood vessels and can be observed in both B-mode and contrast mode, and the average duration of microbubbles in circulation was 6–10 min. When the internal circulation of the microbubbles ends, through the blood flow signal, it can be seen that the blood flow signal weakened and changed direction in some parts, which may be related to the formation of turbulence in the thrombosis area. After the successful construction of the thrombus model, we recorded both B-mode and contrast-mode images 10 minutes for four groups: thrombus + no microbubbles, thrombus + cRGD-MBs, thrombus + GPI + cRGD-MBs and thrombus + non-targeting microbubbles. The results showed that cRGD-MBs showed significant the signal intensity in both B-mode and contrast-mode imaging than other groups(Fig. 3C). In contrast-mode, due to the high frequency of the probe (40MHz) and the use of linear contrast mode, the process of microbubbles filling the blood vessels and the final enhancement of signals in the lumen are not so obvious, but still show a trend of signal enhancement except of the thrombus site. Through the calculation of gray value, we found that whether in B-mode or contrast-mode, the increase of gray value in mice injected with cRGD-MBs was significantly higher than other groups (Fig. 3D).

**Fluorescence imaging of in situ thrombotic animal model**

To observe thrombus-specific fluorescent imaging of cRGD-MBs, thrombotic models were induced using FeCl₃ soaked filter paper on the exposed left distal common carotid artery (CCA). The fluorescence signal of the thrombus was detected using a small animal fluorescence imaging system at different time points (pre-injection, 5min, 10min, 15min, 30min, 60min, and 120 min). The results showed that in the sham-operation group, no strong fluorescence signal could be obtained at any time point, whereas in the thrombus model group, we could get increasing thrombus signals at the common carotid artery of mice, and the fluorescence signal intensity reached a maximum value around 30 min. In the thrombus group with the antagonist tirofiban, we could also obtain an obvious fluorescence signal in the carotid artery of mice, and the intensity changed with time lapse (Fig. 4A). This indicates that cRGD-MBs could bind to
activated platelets and generate fluorescence signals at the lesion site in the presence of thrombus lesions and activation platelets, but with a weak binding ability to inactivated platelets. Moreover, cRGDfK was the key factor for microbubbles binding to thrombus, because in the non-targeting microbubbles group, we could only detect the weak fluorescence signal at 5min and 10min after the injection of the microbubble, and the fluorescence signal declined rapidly. During the period of the cycle in organisms with cRGD-MBs, we can observe in sham-operation and non-targeting microbubble group, the fluorescence signal intensity was basically at a very low level. In the thrombus group, the fluorescence signal showed a trend of change over time, but from the perspective of the fluorescence intensity of the whole, the fluorescence intensity of the thrombus group was 1.3 times that of the antagonist group (Fig. 4B). These results indicate that in the process of thrombosis, cRGD-MBs can be enriched in the position of the thrombosis because the activation of platelets, and tirofiban (GPIIb/IIIa antagonists) can reduce this process, proving that cRGD-MBs have a good targeting effect on activation of GPIIb/IIIa. There was almost no obvious fluorescence signal in the sham operation group, indicating that cRGD-MBs have weak affinity to inactivated platelets. Therefore, it has been proved that cRGD-MBs as a contrast agent can effectively target thrombus and has an excellent imaging ability.

Histological staining and contrast biodistribution toxicological analysis

H&E-stained sections of CCA from an in situ thrombotic mouse model showed a distinct thrombus in CCA tissues following FeCl₃ exposure. Immunofluorescence staining of CCA tissues that the green fluorescence signal of FITC-labeled CD62 antibody at the thrombus was clear and co-located with the red fluorescence signal of cRGD-MBs (Fig. 5A). Through semi-quantitative analysis of immunofluorescence results, we found that the fluorescence signal of the thrombus decreased, especially for cRGD-MBs, in tirofiban-pretreated group (Fig. 5B). The results of fluorescence imaging of major organs of mice showed that microbubbles were mainly enriched in the liver, and a small amount existed in the kidney and lung (Fig. 5C). We have confirmed this through quantitative analysis, which may represent the metabolic pathway of microbubbles in animals (Fig. 5D). In addition, we collected tissues from animals in the thrombus model and performed histological analysis of major organs to determine the biocompatibility of microbubbles. The results showed that the major organs had no histological abnormalities, such as tissue injury or inflammation (Fig. 6A).

Discussion

Thrombus formation in the coronary artery is a leading pathological cause of death in patients with myocardial infarction. The early detection of a thrombus is still a clinical challenge with high theranostic significance. Contrast-enhanced ultrasound is expected to provide specific and individual theranostic approaches for various cardiovascular diseases with fewer side effects. In the present study, we conjugated a cyclopeptide cRGDfK targeted microbubble (cRGD-MBs), which is specific for the activated GPIIb/IIIa receptor on platelets, to provide a novel ultrasonic/fluorescence dual-mode contrast agent for monitoring the thrombus effectively in vivo.
Our data demonstrate that targeted microbubbles cRDG-MBs can selectively bind to activated platelets and thrombosis, thereby allowing molecular ultrasound imaging of thrombosis. Considering the selection of diagnostic targets for thrombus, platelet integrin glycoprotein GPIIb/IIIa undergoes a conformational change upon platelet activation, which makes the activated conformation of GPIIb/IIIa a unique target for the detection of activated platelets. In addition, the fact that GPIIb/IIIa receptor is highly abundant on platelets (60,000 to 80,000 receptors/platelet) and has low expression on circulating platelets in vessels, makes it an ideal target for molecular imaging.\cite{15} Peter et al. previously generated specific single-chain antibodies (scFv) for targeting activated GPIIb/IIIa and showed their unique suitability for the imaging of activated platelets by various imaging modalities such as ultrasound and MRI.\cite{16,17} In our study, we selected cRGDfK as the RGD-containing peptide for targeting activated GPIIb/IIIa. cRGDfK can be modified in many approaches, which is more conducive to investigate the mechanism. Although various RGD-containing peptides have been used to target thrombus, but according to previous study, the conformation of the RGD backbone, the spatial orientation of the charged Arg and Asp side chains, and the role of the hydrophobic moiety flanking the Asp residue could affect the binding ability of peptides to GPIIb/IIIa, and RGD peptides containing d-Phe may have higher biological activity. Olga Kononova et al. verified that cRGDfK and the γc-12 of fibrinogen γ chain had the same binding ability to GPIIb/IIIa by optical trap-based nanomechanical measurements and computational molecular modeling. As a micromolecular polypeptide, cRGDfK has the following advantages: strong selectivity, low immunogenicity, and good biocompatibility, so it may have a good potential in the clinical application of thrombus diagnosis in the future. Moreover, cRGDfK is the most widely used in the study of integrin αvβ3, but as far as we know, it is rarely used in the study of GPIIb/IIIa and thrombus detection. To compare the binding ability of traditional RGD-MBs and cRGDfK-MBs we prepared to GPIIb/IIIa and activated platelets, we further compared previous studies with ours.\cite{15} Rekha Srinivasan et al. constructed a liposome containing CRGDC and analyzed the adhesion efficiency of liposomes to activated platelets by flow cytometry.\cite{18} In our study, we similarly quantified the adhesion efficiency of cRGDfK-MBs to activated platelets by flow cytometry. Comparing the change in mean fluorescence intensity when two RGD-containing microbubbles bound to resting platelets and activated platelets, we found that cRGDfK-MBs had a greater affinity for activated platelets and were more able to distinguish activated platelets from resting platelets. These results indicated that cRGD-MBs may have a stronger affinity for thrombus and the potential to provide rapid and accurate diagnosis and targeted treatment in clinical thrombotic diseases.

There are many clinically available imaging techniques for thrombus detection, including ultrasound. The common features of ultrasound include real-time, noninvasive, high safety, and wide availability. The ultrasound contrast agent enhanced the signal of the blood pool and improved the accuracy and credibility of the disease diagnosis.\cite{19–21} There are many kinds of ultrasound contrast reagents targeting different molecular targets, which have achieved good imaging results in molecular imaging studies of diseases such as atherosclerosis or thrombosis.\cite{22–24} Compared with ultrasound imaging, fluorescence imaging has the characteristics of high sensitivity and has become a powerful tool for molecular imaging.\cite{25–27} Our previous study has reported that OPN-targeted ultrasound/optical dual-modality
nanoparticles could provide in vivo imaging for vulnerable atherosclerotic plaques. Near-infrared fluorescent imaging is more suitable for in vivo disease diagnosis because of its strong penetrating power. Therefore, near-infrared fluorescence/ultrasound dual-modality imaging has great potential for clinical application with good spatial resolution of ultrasound imaging and the high sensitivity of fluorescence imaging.

In the present study, for thrombosis diagnosis, we selected activated GPIIb/IIIa on platelets as the target of ultrasound microbubbles. The microbubbles were fabricated using a C$_3$F$_8$ gas core with a shell composed of DSPC, DSPE-PEG-cRGDFK, and fluorescent dye DiD. Concerning the design of microbubbles, PEG prevents coalescence and can stabilize microbubbles, so it was incorporated into the shell as a space stabilizer. Coupling of peptides to DSPE-PEG-2000 is a mild and high-yield process for the covalent binding of peptides to lipids. Liposome was prepared by thin-film dispersion and ultrasound microbubbles were built by the mechanical oscillation method. During high-frequency mechanical oscillations, different positive and negative pressures are generated, where the negative pressure can cause the gases present in the preparation fluid to form tiny bubbles. Because C$_3$F$_8$ has high molecular weight, poor solubility and slow diffusion, it can't diffuse easily from the microbubbles into the solution and can form stable microbubble. The concentration of microbubble was determined to be $1.02 \times 10^9$ (MBs/ml) by analysis. Using a mouse common carotid artery thrombosis model, we found that the fluorescence signal achieved the accurate location of carotid thrombus. In particular, by ultrasound imaging, we can determine the site of thrombosis through the change of blood flow signal, and we observed an enhancement of the ultrasound signal in the corresponding position. Moreover, the change of the signal can be monitored in real time, which further proves the contrast agent for thrombosis have well targeting and in vivo imaging capability. Hu et al. have reported a similar microbubble containing RGD peptide that can specifically bind to thrombus and achieve imaging only by ultrasound imaging. Compared with traditional microbubbles, the ultrasound microbubble contrast agent in this study has the dual ability of both fluorescence imaging and ultrasound. Fluorescence imaging has high sensitivity and is more conducive to the study of the mechanism, but there are some limitations as well. For example, we found some non-specific binding of non-targeted microbubbles in ultrasound imaging. Although the increase in ultrasound signal from non-targeted microbubbles was weak, but in fluorescence imaging, no obvious signal was detected in the non-targeted group. Combining the results of multiple replicate experiments, we considered that the non-specific binding was insufficient to allow us to collect a strong enough fluorescence signal, so there was nothing detected in fluorescence imaging of non-targeted group. On the other hand, ultrasound imaging has high sensitivity on detecting individual microbubble, so that ultrasound can find some off-target binding. These results revealed that the selection of fluorescent dyes with stronger tissue penetration and safe for the human body is one of the key points that we need to improve in the future. For example, ICG can be used to replace DiD in the future, making it possible to conduct ultrasound/fluorescence bimodal imaging in the human body, to realize real-time, dynamic and highly sensitive imaging of thrombus.
In our *in vivo* imaging study, we used a 40MHz ultrasound probe to facilitate the observation of superficial mouse carotid arteries. For *in vitro* experiment imaging of ultrasound microbubble, we used the thyroid ultrasound probe in clinic, with the frequency of 3-12Hz, we found that although under low frequency probe, the microbubble can still produce a good imaging effect. Moreover, the low frequency probe can detect deeper tissue, and low mechanical index can reduce the damage to the microbubble, Therefore, we believe that the microbubble prepared by us may have a good prospect in the future clinical application.

It is well known that microbubbles are avidly taken up by spleen.\[^{36}\] We considered that no fluorescence signal was observed in the spleen in our study because sampling was performed 2 hours after injection of microbubbles and the mice were perfused with paraformaldehyde. Therefore, we obtained the major organs of mice 15 and 30 minutes after microbubble injection, respectively. We found that at 15 min after microbubble injection, the fluorescence signal was strongest in the lungs, which is due to the destruction of microbubbles mainly in the lungs. At 30 min, the signal was weakened in the lungs and enhanced in the spleen and liver, because microbubbles are easily enriched in the spleen and the liver is also an important metabolic organ.

This study has some limitations. First, the thrombus animal model by chemical damage cannot simulate the thrombosis caused by plaque rupture in humans. The thrombus model caused by chemical injury is an oxidative damage model from the adventitia to the intima, which is not exactly the same as the thrombus mechanism of atherosclerotic plaque rupture. \[^{37,38}\] Second, in the *in vitro* imaging experiment, we used nonlinear contrast imaging to evaluate the imaging ability of the ultrasound contrast agent, whereas for the *in vivo* imaging, linear contrast was chosen because of technical limitations. Contrast agent signal was affected by the tissue background, so that the enhancement of the thrombus signals was less robust, which needs to be improved in future experiments.

**Conclusion**

cRGD-MBs have a high affinity for activated platelets and can monitor fresh thrombus formation *in vivo* via ultrasound/fluorescence dual-mode imaging. This dual-modality-based molecular noninvasive imaging technique will provide an ideal tool for the real-time early diagnosis of thrombotic diseases in future clinical applications.

**Declarations**

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All animal studies and procedures were performed according to the protocol approved by the Chinese PLA General Hospital Animal Care and Use Committee in accordance with the Care and Use of Laboratory Animals formulated by the National Society for Medical Research.

Conflict of Interest

The authors declare that they have no conflict of interest.

References


Figure 1 Characterization of cRGD-microbubbles. a) Schematic illustration of ultrasound microbubbles and the structural formulas of DSPE-PEG-cRGDFK, DiD, and DSPC; b) TEM image of cRGD-liposome; c) Fluorescence images of cRGD-MBs where red fluorescence represented the DiD; d) Normalized emission spectra of cRGD-MBs; and e) Size distribution of cRGD-MBs.

Figure 1

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Figure 2

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