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COMMUNICATION

Click chemistry-mediated rapid microbubble catching for acute thrombus ultrasound molecular imaging

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Abstract: Bioorthogonal coupling chemistry has been studied as one significant advantage for molecular imaging as it offers rapid, efficient, and strong binding, which may also benefit in stability, production, and chemical conjugation. The inverse-electron-demand Diels-Alder reaction between s-tetrazine and trans-cyclooctene (TCO) is an example of a highly selective and rapid bioorthogonal coupling reaction to be used successfully to prepare targeted molecular imaging probes. Herein, based on a two-step pretargeting bioorthogonal chemistry, we report a fast and reliable highly sensitive approach to achieve activated-platelet-specific CD62p targeted thrombus ultrasound molecular imaging. Tetrazine modified microbubbles (Tetra-MBs) could be uniquely and rapidly captured by subsequent click chemistry of trans-cyclooctene tagged CD62p antibody pre-treated thrombus. Moreover, such Tetra-MBs showed great long-term stability under physiological condition to maintain the ability to monitor thrombus changes in real-time. We demonstrated for the first time that Tetra-MBs based bioorthogonal targeting molecular ultrasound imaging strategy could be a simple but powerful tool for rapid diagnosis of acute thrombosis.

In order to fabricate molecular imaging probe for specific molecular imaging modality, one of the major conventional targeting strategies is to conjugate specific ligands on the surface of contrast agents for binding to specific receptor of tissues based on both non-covalent (such as electrostatic interactions,[1,2] hydrophobic attractions,[3] or avidin-biotin[4,5]) and covalent bonding.[6,7] Recent years, bioorthogonal coupling chemistry has been studied as one significant advantage for molecular imaging as it offers rapid, efficient, and strong binding, which may also benefit in stability, production, and chemical conjugation.[8-10] The inverse-electron-demand Diels-Alder reaction between s-tetrazines and trans-cyclooctene (TCO) is an example of a highly selective and rapid bioorthogonal coupling reaction to be used successfully to prepare targeted nuclear and optical molecular imaging probes.[11-13] By using a bioorthogonal reaction, pre-targeted molecular imaging strategy has become a powerful tool for in vitro and in vivo imaging under different modalities.[14] In a typical bioorthogonal pretargeting approach, the target is first bound by a TCO modified antibody, and then a tetrazine probe is introduced in order to achieve labeling through the cycloaddition reaction.

Thrombosis is developed due to blood vessel wall damage or pathological process that induces activation of coagulation factor to cause accumulation of platelets and fibrin.[15] Although many imaging strategies including magnetic resonance imaging (MRI),[16] ultrasound (US),[17] computed tomography (CT),[18] etc. have been developed to visualize thrombus formation process, the specific detection and rapid evaluation of thrombotic events, especially at the molecular level, presents a major clinical challenge. US is a clinical diagnostic imaging modality that is widely available, portable, and non-invasive for visualizing anatomy and various physiology.[19] Contrast-enhanced ultrasound using gas-filled micro-sized bubbles (MBs, 1~10 μm) has further enhanced the utility of ultrasound and proved to be an effective imaging contrast agent to more specifically image blood flow, discern the location of certain molecular targets.[20,21] As one of the thrombus formation imaging tools, contrast-enhanced ultrasound has been proved to be very effective if the targeting microbubble concentration attached to the clot is enough in the short time. However, the low affinity and short retention time attached to the thrombus based on the traditional steady binding under the blood flow significantly weaken the targeting efficiency and imaging effect.[22] Herein, pretargeting strategy for acute thrombus molecular detection based on bioorthogonal reactions have been investigated. As shown in Scheme 1, firstly, TCO modified CD62p antibody (TCO-antiCD62p) was synthesized and pre-injected. CD62p antibody was an important targeting probe to adhesive receptors present on platelets (P-selectin). Upon activation of platelet to form thrombus, fusion of the granules with the external membrane exposes P-selectin at the platelet surface.[23,24] Thus, at first step, treated with TCO-antiCD62p, thrombus can non-covalently bind and interact with TCO-antiCD62p. Secondly, an elaborately fabricated s-tetrazine-tagged microbubbles (Tetra-MBs) were added, following by rapidly and selectively reaction with TCO-

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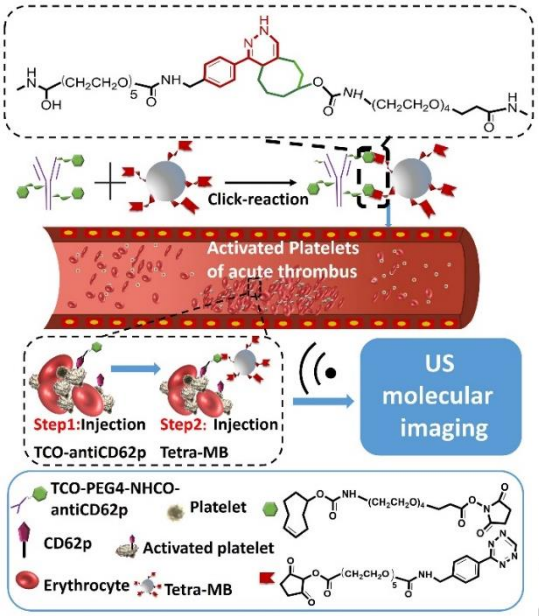
antiCD62p pre-treated thrombi or platelet aggregations, which imaged by real-time US imaging. Due to the microbubble's high spatial and temporal sensitivity, such pre-targeting strategy provides thrombus with high spatial precision for early formation detection at molecular level. By using a bioorthogonal reaction in this manner, Tetra-MBs are able to be as specific, rapid, and sensitive contrast agents for US imaging of acute thrombosis.



Scheme 1. Schematic diagram of click reaction through pre-targeting and bioorthogonal chemistry between tetrazine-functionalized microbubbles (Tetra-MBs) and an intravascular target (CD62p) labeled with a TCO modified antibody. Fast localizing Tetra-MBs to thrombi can be imaged by ultrasound imaging in real-time.

The DSPE-PEG_{2k}-NHCO-PEG_5-Tetrazine was synthesized via peptide coupling from Tetrazine-PEG_5-NHS and DSPE-PEG_{2k}-NH_2 bearing an amine-reactive linker (Figure 1a). The obtained DSPE-PEG_{2k}-NHCO-PEG_5-Tetrazine compound was purified and characterized using reversed-phase C18 high-performance liquid chromatography (HPLC). Figure 1b (circle box) indicated that retention time of DSPE-PEG_{2k}-NH_2 and DSPE-PEG_{2k}-NHCO-PEG_5-Tetrazine were 8.35 and 8.85 min, respectively, which confirmed that the tetrazine molecules were conjugated with DSPE-PEG_{2k}-NH_2 lipid.

Based on the DSPE-PEG_{2k}-NHCO-PEG_5-Tetrazine lipid, the Tetra-MBs were prepared and characterized. The submicron-sized bubbles were removed from the solution by flotation at 300× g for 3 min. After 3 washes, a major peak was observed in the diameter distribution of the remaining MBs at 1.76 μm. Quantification of microbubble concentration using a Multisizer (4e, Particle Sizing System, Santa Barbara, USA) indicated about 1-5×10^9 microbubbles mL^{-1} of solution. The optical microscopy results showed the spherical morphology of the Tetra-MBs (Figure 1c).

The TCO-antiCD62p was constructed via reaction of the antibody with 10 molar equivalents of the N-hydrosuccinimidyl ester of TCO (TCO-PEG_4-NHS) for 30 min at room temperature (RT) in PBS adjusted to pH 8.8-9.0 with $NaHCO_3$ (0.1 M) (Figure 1d). The extended PEG_4 spacer of TCO may reduce aggregation, minimize steric hindrance, and enhance solubility, which is beneficial for the conjugation between TCO and antiCD62p proteins.[25] The TCO-antiCD62p bioconjugates were purified using centrifugal filtration. An average of about 3 to 4 TCO molecules were conjugated to one antibody, as determined by MALDI-TOF mass spectrometry (Figure S1, Supporting Information). Moreover, the agarose gel electrophoresis results in Figure 1e suggested the TCO-PEG_4-NHS were bioconjugated with CD62p antibody.

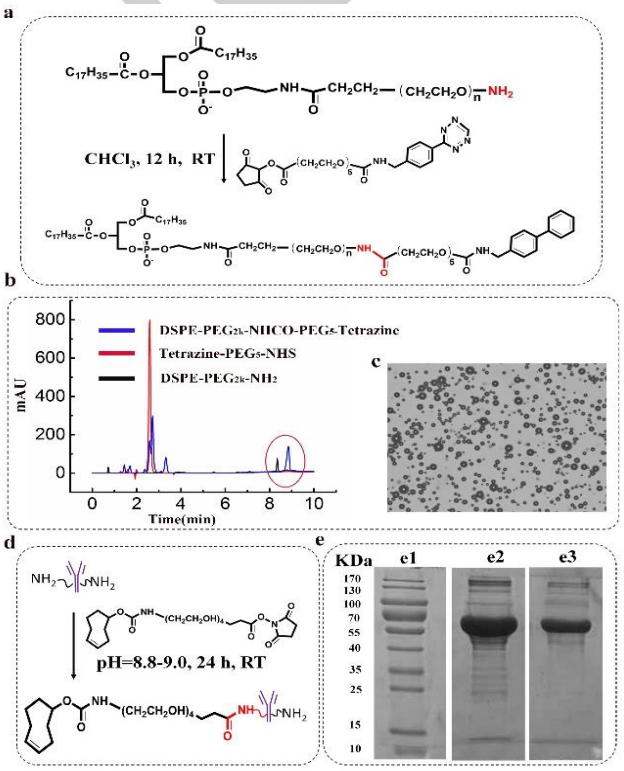


Figure 1. (a) DSPE-PEG_{2k}-NHCO-PEG_5-Tetrazine lipid synthetic scheme showing reaction between tetrazine-PEG_5-NHS and DSPE-PEG_2k-NH_2. (b) The HPLC results of tetrazine-PEG_5-NHS, DSPE-PEG_{2k}-NH_2, and DSPE-PEG_{2k}-NHCO-PEG_5-Tetrazine. (d) Optical microscope images of tetrazine modified microbubbles (Tetra-MBs). (d) TCO-antiCD62p synthetic scheme showing reaction between CD62p antibody and TCO-PEG_4-NHS. (e) Agarose gel electrophoresis results of CD62p antibody and TCO conjugated CD62p antibody: (e-1) Standard protein, (e-2) antiCD62p, and (e-3) TCO-antiCD62p.

With the 2 system components in hand, we next investigated the reaction between TCO-antiCD62p and Tetra-MBs. Firstly, Cy3-tetrazine was used to explore TCO-antiCD62p reactivity after conjugation on the US-activated platelet (Figure S2, Supporting Information). Cy3-tetrazine was added to the activated platelet solution that had been pre-incubated with TCO-antiCD62p. Results in Figure S3 (Supporting Information) showed that about 98% TCO-antiCD62p antibodies pre-treated platelets can

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specifically bind with Cy3-tetrazine within 5 min, which confirms that the pretargeting strategy based on TCO-Tetrazine bioorthogonal reaction is rapid and high efficiency even when TCO was bioconjugated with CD62p antibody.

Moreover, we then evaluated the effectiveness of the Tetrazine-TCO capture strategy after tetrazine molecules were conjugated on the surface of MBs. The targeting experiment of Tetra-MBs were evaluated in vitro under flow conditions (300 s^{-1}) simulating to the in vivo blood flow by using a parallel-plate flow chamber system (Glycotech, Rockville, Md.). Results in Figure 2a indicated the dynamic optical microscopic observation of Tetra-MB attachment process during the Tetra-MB flowing (5 min) and after following 5 min PBS washing. The optical microscopy clearly indicated that the Tetra-MBs could attach to (red circles in Figure 2a). Quantitatively, Figure 2b exhibited that there was about 17% Tetra-MBs specifically bound with by the platelet aggregations after PBS washing. However, as shown in Figure S4 (Supporting Information), a relatively small amount of Tetra-MBs and control MBs bound non-specifically to the platelets without TCO-antiCD62p pre-treatment during the flow chamber dynamic assays, which all of them were removed after the final washing step. Interestingly, compared with MBs with antiCD62p directly coupled on the surface, there were more than 2.4 times microbubble amount localized on the activated platelets for pre-targeting Tetra-MBs. Thus, it is indicated that the conventional targeting MBs with antiCD62p coupled on the surface demonstrated lower targeting efficiency with activated platelets than Tetra-MBs. All these data establish that the Tetrazine-TCO cycloaddition approach used for microbubble catching for TCO tagged activated platelets is sufficiently rapid and chemo-selective efficiency. Figure 2c further confirmed that the Tetra-MBs can specifically target and be captured by US-activated platelet aggregation and imaged by real-time US imaging.

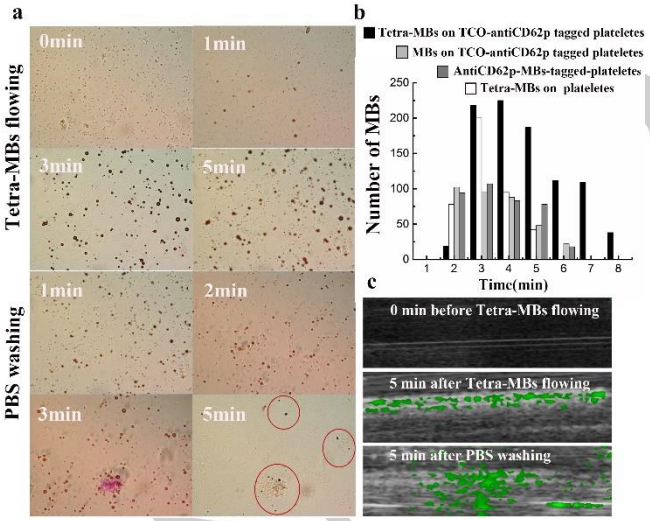

Figure 2. (a) The Tetra-MBs dynamic attachment to platelets was captured at the time point of 0, 1, 3, 5 min when Tetra-MBs flowing and after PBS washing at the time point of 1, 2, 3, 5 min. Red circles indicated the bubble targeted to platelet aggregates. (b) The quantitative number analysis of MBs conjugated with platelets in vitro. (c) Ultrasound imaging of the Tetra-MBs catching process over time.

We continued to assess the in vivo dynamic targeting efficacy of Tetra-MBs in rat ferric-chloride-induced inferior vena cava (IVC) thrombosis using US imaging. The ferric chloride model generated venous thrombi identical in size because of the similar dimensions of the filter paper (5 mm ×10 mm) saturated with 10% $FeCl_3$, which was shown in Figure S5a. The venous blood flow determined by US pulsed-wave Doppler before and after thrombosis were 118±8.3, 217±11.5 mm s^{-1}, respectively (Figure S5b, Supporting Information). According to the protocol in Figure S5c (Supporting Information), the thrombus-bearing rats were divided into three groups and injected with Tetra-MBs with and without anti-CD62p pretreatment, as well as control (1×PBS) solution (approximately 1 × 10^5 MBs). The real-time US imaging of administrated rats in each group were then monitored for 2 h. Figure 3a is the typical thrombus US images, which indicates the enhanced US imaging can be observed immediately after microbubble injection. Compared with Tetra-MBs in the absence of TCO-antiCD62p pretreatment, the stable imaging enhancement was observed in those with TCO-antiCD62p pre-treated thrombus group. The rapid Tetra-MBs accumulation in the thrombus can maintain imaging contrast ability ranging from 5 min to 30 min (Figure 3c) and following within the next 2 h timeframe (Figure 3b). After Tetra-MBs injection, echo intensity of US in Tetra-MBs with antiCD62p pre-treated group became much brighter than Tetra-MBs without TCO-antiCD62p pre-treated group. Our data demonstrates that these targeted Tetra-MBs can selectively bind to thrombi, thereby allowing successful molecular ultrasound imaging of thrombosis. Ultrasound images acquired 5 min thereafter showed high retention of the microbubbles in activated platelet regions of the thrombi maintained significant contrast enhancement compared to rat without TCO-antiCD62p pretreatment. Thus, even under the physiological blood flowing (217±11.5 mm s^{-1}), the boundaries of the thrombus in the vessels can be clearly visualized by the existence of stable MBs. The significant difference between these two groups could be attributed to the rapid ability of two bioorthogonal reaction pairs using Tetrazine-TCO, which ensures high targeting accumulation concentration of MBs to the freshly formed clot even under the high shear stress blood flow.

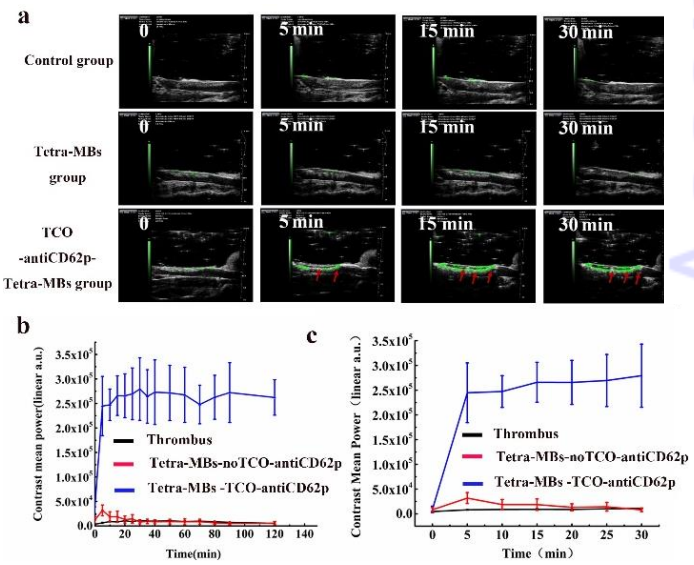

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Figure 3. (a) US imaging of the thrombus area of ferric-chloride-induced inferior vena cava (IVC) thrombosis rats during the tail intravenous injection of PBS solution, with Tetra-MBs without and with antiCD62p pretreatment. And quantitatively, the average mean gray time-course for 2 h (b) and within 30 min (c).

Histology of the thrombosis was observed in the hematoxylin and eosin (HE) stained section under the bright field microscope. Figure 4c confirmed that the scattered microbubble-likes shapes were seen around the intraluminal thrombus and inside the cellulose of the thrombus for the group of Tetra-MBs injection of TCO-antiCD62p pretreatment. The MBs were irregular in shape, and some of them were either deformed or ruptured. During the thrombus formation, the pretargeting strategy in Figure 4c group ensured that the Tetra-MBs were rapidly captured and accumulated by the activated platelets (imaged in Figure 3), which prevented the further aggregation of platelets on the clot. Thus, the Tetra-MBs on the surface of activated platelets of the clot were observed. Result in Figure 4 further confirmed that the Tetra-MBs can be retained in the thrombus tissue for US imaging and future therapy.

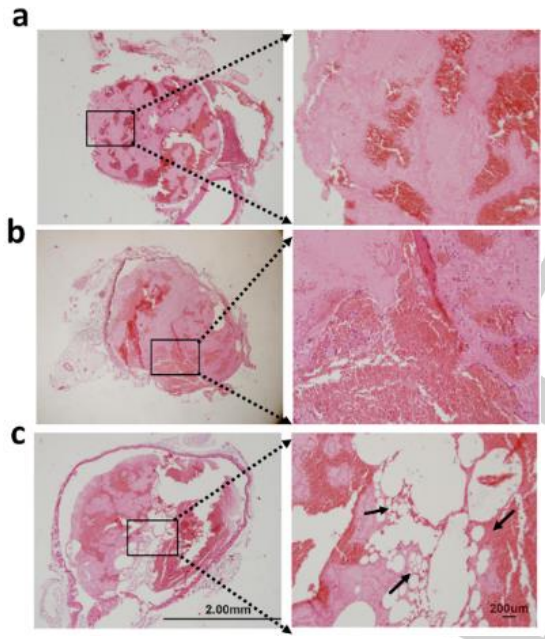

Figure 4. Representative optical images of hematoxylin and eosin stained thrombi in the IVC thrombus-bearing rats obtained after US imaging injected with PBS solution (a), with Tetra-MBs without (b) and with anti-CD62p pretreatment (c). The black arrows indicate the existence of microbubbles in the thrombi.

In conclusion, we have provided the successful evidence that capturing MBs in vitro and in vivo is feasible using bioorthogonal tagging two-step pretargeting approach for acute thrombosis. Tetrazine modified MBs can specifically and rapidly bind to the activated-platelet-specific CD62p targeted thrombus for facilitating ultrasound molecular imaging. The ability of Tetra-MBs to show click reaction upon TCO-antiCD62p bioconjugates is a major advantage especially for applications where MBs washout is not desired for long-term US imaging during thrombus formation. This study opens a door for developing many innovative strategies for diagnosis, prevention and treatment of acute thrombus in the future. Additional experiments are required to investigate the pharmacokinetics and pharmacodynamics of the targeted Tetra-MBs.

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Keywords: click chemistry • imaging agents • molecular devices •p-selectin • ultrasound

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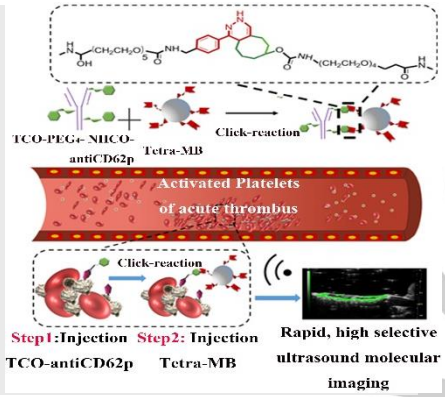

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