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Multifunctional Thrombin-Activatable Polymer Capsules for Specific Targeting to Activated Platelets

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Hemostasis is a key biological feature that prevents sustained bleeding upon injury.[1] Acute thrombosis, a leading cause of mortalities and morbidities worldwide, is a pathological hemostatic condition, which is mainly caused by the rupture of an unstable atherosclerotic plaque leading to myocardial infarction or stroke.[1-2] These unwanted intravascular blood thrombi cause vascular occlusions and organ damage due to prolonged lack of oxygen.[1b] As a first line of defense, fast-acting thrombolytic drugs, such as plasminogen activators (PAs), are administered to stop thrombus growth and to quickly induce thrombus deconstruction.[3]

However, systemic (intravenous) or local (catheter) delivery of PAs is deemed neither effective nor safe due to either rapid neutralization by their antidotes (e.g., anti-plasmin) or bleeding complications that may lead to intracranial hemorrhages.[1] With no safe thrombolytic treatments currently available and a lack of timely treatments, acute thrombosis remains a major healthcare issue contributing to a significant number of deaths and disabilities.

The pressing need for a therapeutically efficacious and safe strategy has led to the development of state-of-the-art carrier designs[4] that exploit the intrinsic properties of thrombosis for targeting specific receptors and/or triggering payload release.[5] These carriers can deliver the thrombolytic drugs to specific sites, hence minimizing adverse side effects. Platelet activation, which induces the formation of a platelet-rich thrombus,[1] is a hallmark of thrombotic events. It involves the mobilization and conformational changes of the surface-bound integrin receptors that can be used to target activated platelets (via RGD peptides[6] or anti-glycoprotein (GP) IIb/IIIa antibody[7]) or fibrin (via anti-fibrin antibody[7a]). The blood coagulation cascade, in parallel, creates a distinct microenvironment[1b, 8] and produces a large amount of thrombin that converts fibrinogen to fibrin, that is, a biological/mechanical sealant for the platelet aggregates.[1b,8] These insights have advanced research in the design of systems that respond to these microenvironments.[9] However, such systems either lack efficient cargo encapsulation, active targeting components, and/or require external triggers for payload release.

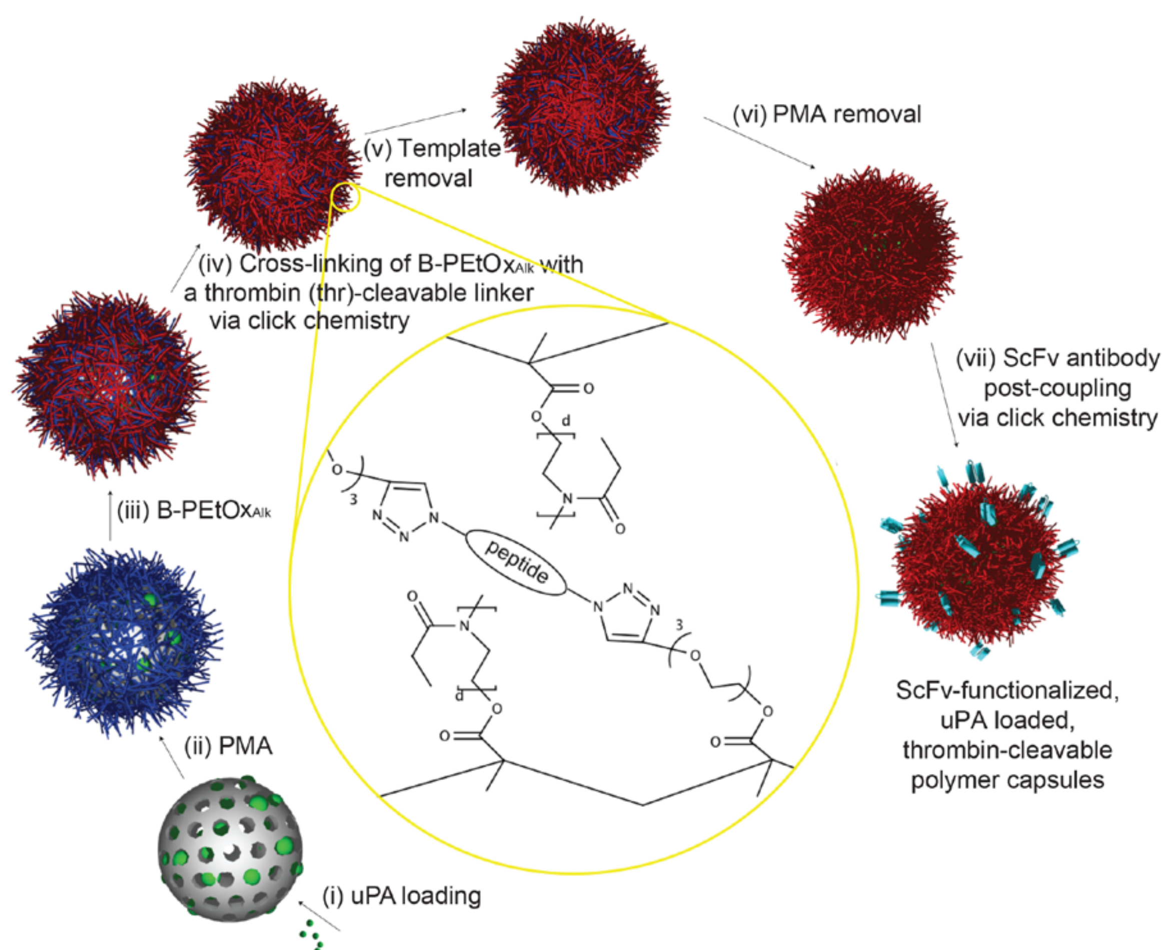
Herein, we report a multifunctional (i.e., antibody-targeted, thrombin-cleavable (thr-cleavable) and urokinase plasminogen activator (uPA)-loaded) polymer carrier system that responds to the thrombus microenvironment to initiate thrombolysis. The carrier targets the active form of GPIIb/IIIa integrin receptors on activated platelet surfaces and is cleavable by the serine protease thrombin to selectively trigger the release of the thrombolytic agent, that is uPA, for fibrin degradation.[3,6] Sensitivity to thrombin was introduced by integrating a 10-

mer peptide sequence (ELTPRGWRLE) consisting of thrombin recognition sites.[10] This sequence was shown to have similar thrombin-cleavable efficiency to a phage-display derived peptide,[10d] and glutamic acid (E) was added to both ends of the peptide to provide an overall neutral charged sequence to minimize non-specific interactions with other building blocks of the polymer carrier. The peptide contains bisazide end groups and was used as a cross-linker for the carrier. For targeting, we employed a phage-display derived single-chain antibody (scFv) specific for the highly abundant fibrinogen receptor GPIIb/IIIa, but only in its activated form.[5c,11]

The carrier design was based on the layer-by-layer (LbL) assembly[12] of poly(2-oxazoline) (POx)-based materials. POx is a highly functional polymer class[13] and has gained interest as a poly(ethylene glycol) alternative due to its excellent biocompatibility[14] and protein repellent properties.[15] Our recent work on POx-based capsules[16] demonstrated their low fouling behavior and biodegradability. The significance of the current study resides in several aspects: (i) the use of MS particles as templates to provide efficient encapsulation of uPA to induce thrombus dissolution; (ii) the use of brushlike poly(2-ethyl-2-oxazoline) with alkyne functional groups (B-PEtOx$_{\mathrm{Alk}}$),[16a] which was shown to be low fouling; (iii) the integration of a thrombin-sensitive cross-linker to activate carrier degradation and cargo release in the area of thrombosis; and (iv) the attachment of scFv on the polymer capsule surface for specific targeting to activated platelets.

Synthesis of the multifunctional polymer capsules was achieved via LbL assembly (**Scheme 1**). The influence of temperature on uPA activity was assessed to ensure that the chosen operating temperatures did not cause any loss of activity (Figure S1). UPA was pre-loaded into MS particle templates (1.4 µm diameter) at 4 °C in PBS (pH 7.4) via electrostatic interactions between the slightly positively charged uPA (pI ~8.7[17]) and the negatively charged MS particles. To quantify the amount of non-adsorbed uPA, the MS particle templates were centrifuged (1100 *g*, 30 s) following Alexa Fluor 488 (AF488)-labeled uPA

loading. The fluorescence intensity of AF488-labeled uPA in the supernatant solution was measured against a calibration curve (Figure S2). In addition, the fluorescence intensity of loaded uPA before and after template removal was compared to account for the uPA loss during this step. An adsorbed amount of 6.4×10^{-15} g uPA (or 2.6×10^{-8} units) per capsule was determined. A colorimetric assay of uPA in the resulting polymer capsules verified that the uPA activity was maintained at this level, based on a calibration curve (Figure S3).



Scheme 1. LbL assembly of multifunctional carriers: uPA-loaded, thr-cleavable, scFv-functionalized B-PEtOx$_{Alk}$ (Scheme S1 for chemical structure) capsules. (i) UPA loading into MS particle templates, (ii) poly(methacrylic acid) (PMA) deposition onto uPA-loaded MS particle templates, (iii) B-PEtOx$_{Alk}$ deposition onto PMA-coated MS particle templates, followed by alternate deposition of PMA and B-PEtOx$_{Alk}$ until the desired number of layers was achieved, (iv) cross-linking of the alkyne moieties of B-PEtOx$_{Alk}$ using thr-cleavable bisazide peptide cross-linker (Scheme S2 for chemical structure) via copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) chemistry in the presence of chelator (Scheme S3 for chemical structure), (v) MS particle template removal using buffered hydrofluoric acid (HF),

(vi) sacrificial PMA layer removal via high pH (PBS, pH 7.4) washing, and (vii) scFv antibody post-coupling onto the B-PEtOx$_{Alk}$ capsule surface via CuAAC chemistry in the presence of chelator.

Poly(methacrylic acid) (PMA) and B-PEtOx$_{Alk}$ were alternately deposited onto uPA-loaded MS particle templates at pH 4, 23 °C via hydrogen bonding. The (PMA/B-PEtOx$_{Alk}$)$_n$ assembly on planar substrates, analyzed by quartz crystal microgravimetry (QCM), and on colloidal templates, followed by flow cytometry, demonstrated film buildup (**Figure 1**a,b). Of note is that LbL assembly did not cause any loss of uPA (Figure S4). The multilayers were cross-linked using a thr-cleavable bisazide cross-linker via copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) click chemistry in the presence of a chelator (Scheme S3) to prevent possible uPA denaturation.[18] The reaction was left to proceed overnight at 23 °C. Sacrificial MS particle templates and PMA layers were then removed by exposing them to a buffered hydrofluoric acid (HF) and PBS buffer (pH 7.4) to disrupt the hydrogen bonding between B-PEtOx$_{Alk}$ and PMA (Figure S5), respectively. Fluorescence and transmission electron microscopy (TEM) analysis of the polymer capsules demonstrated the formation of uPA-loaded, thr-cleavable B-PEtOx$_{Alk}$ polymer capsules (Figure 1c-f).

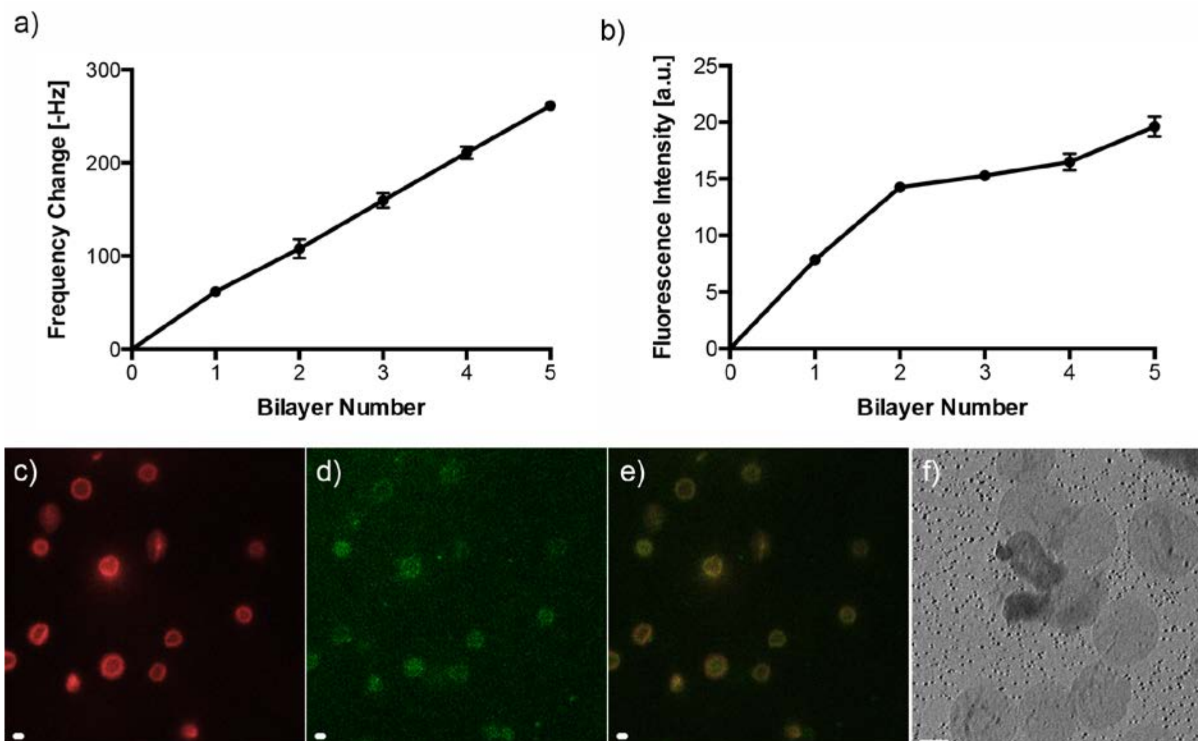


Figure 1. Fabrication and characterization of uPA-loaded, thr-cleavable B-PEtOx$_{Alk}$ polymer capsules. LbL assembly of (B-PEtOx$_{Alk}$/PMA)$_5$ in NaOAc (20 mM, pH 4) on (a) planar substrates, as measured by QCM, and (b) on colloidal supports (MS particles, 1.4 μm-diameter), as followed by flow cytometry (mean ± SD, n = 3). PMA was labeled with AF488-cadaverine for flow cytometry analysis. Measurements were performed after deposition of each PMA layer. Microscopy images of the polymer capsules prepared from 1.4 μm-diameter MS particles: fluorescence microscopy images of (c) AF647-labeled (red) B-PEtOx$_{Alk}$ capsules, (d) AF488-labeled (green) uPA loaded inside the capsules, (e) overlay of a and b, and (f) TEM image of air-dried B-PEtOx$_{Alk}$ capsules. Scale bars are 1 μm.

To investigate the effect of thrombin in inducing capsule degradation and uPA release, uPA-loaded degradable (thr-cleavable) and non-degradable (cross-linked by a d-amino acid sequence, Scheme S2b) capsules were prepared. The loaded capsules were exposed to simulated thrombolytic conditions (50 mM $NaHCO_3$, pH 8)[19] with different thrombin concentrations (0, 0.05, 0.1, and 1 units mL^{-1}).[20] To analyze the degradation and release profiles, the capsules were AF555-labeled (red) and the uPA was AF488-labeled (green). These fluorescence intensities were monitored using flow cytometry at different incubation times of up to 24 h (**Figure 2**). Figure 2 showed that the fastest kinetics were achieved after 4 h with 1 unit mL^{-1} thrombin. Exposure to higher thrombin concentrations (5 or 10 units mL^{-1}) resulted in almost instantaneous (within 15 min) capsule degradation/cargo release kinetics

(Figure S6). The concentration-dependent kinetics of the degradable variants verify the thrombin-specific activation feature of this carrier, as shown by thrombus dissolution (at 4 h) induced only by degradable capsules (Figure S7). In contrast, Figure S6 and S8 showed that the non-degradable counterparts had no effect on thrombin (up to 10 units mL^{-1}), indicating the thrombin specificity of the thr-cleavable variants.

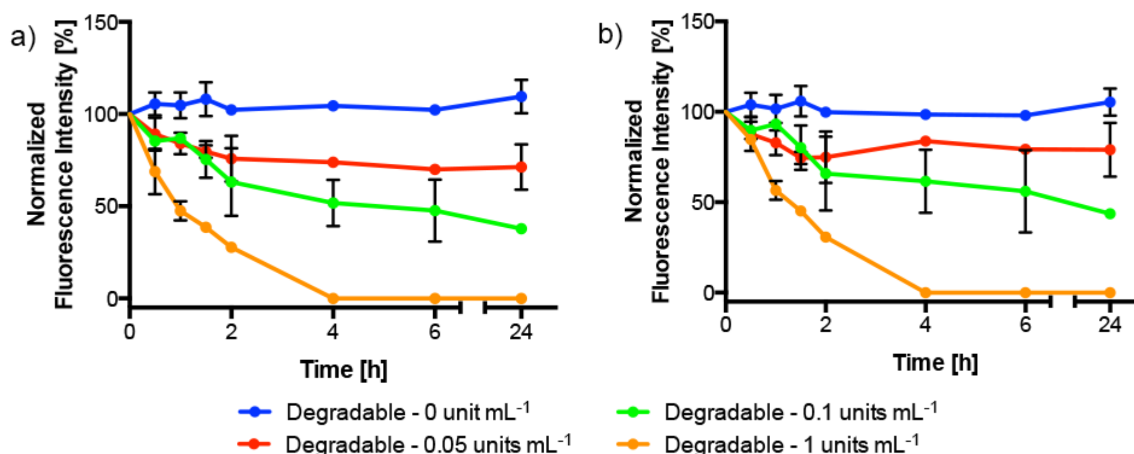


Figure 2. Enzymatic degradation profiles of uPA-loaded B-PEtOx$_{Alk}$ capsules. (a) Degradation of AF555-labeled (red) B-PEtOx$_{Alk}$ polymer capsules, and (b) release of loaded AF488-labeled (green) uPA. Capsules cross-linked by a thr-cleavable (degradable) cross-linker were subjected to 0 unit mL^{-1} (blue), 0.05 units mL^{-1} (red), 0.1 units mL^{-1} (green), or 1 unit mL^{-1} (orange) of thrombin. The reactions were performed in $NaHCO_3$ (50 mM, pH 8.0) at 37 °C, with constant shaking (mean ± SD, n = 3).

To incorporate the anti-GPIIb/IIIa receptor targeting functionality, the scFv antibody was coupled with an azide group[21] and conjugated to the B-PEtOx$_{Alk}$ capsule surface. The conjugation was performed via CuAAC chemistry in presence of a chelator (Scheme S3) to avoid structural denaturation of the antibody.[18] The amount of chelator required to retain the scFv function was found to be four times the amount used in our earlier work[18] (data not shown). The conjugation of scFv to B-PEtOx$_{Alk}$ capsules was studied in more detail. The non-specific interactions between scFv and capsule surfaces in the absence of copper(II) were found to be minor (Figure S9a,c,e). On the contrary, the addition of copper(II) resulted in a fluorescence intensity shift, as shown by flow cytometry (Figure S9b). The successful covalent attachment of scFv to the capsules was verified by uniform green fluorescence

(Figure S9d) distributed around the polymer capsules (Figure S9f). To quantify the amount of unreacted scFv, the capsules were centrifuged (2800 *g*, 8 min) and the fluorescence intensity of the supernatant solution was measured using a calibration curve (Figure S10). The amount of scFv attached to the capsules was found to be 9.3×10^{-14} g scFv (1.75×10^{6} scFv molecules) per capsule.

To investigate the GPIIb/IIIa targeting ability, non-loaded, non-degradable capsules, coated with scFv were prepared. Targeting (scFv +ve) and non-targeting (scFv -ve) capsules were prepared with the platelet targeting scFv and the mutated sequence, respectively. To assess scFv affinity on activated platelets, experiments in static (non-flow) and flow chamber settings were performed.

In the static setup, three groups of human platelet-rich plasma (PRP) were prepared: (i) non-activated (PRP only); (ii) activated (PRP treated with adenosine diphosphate (ADP)); and (iii) blocked (activated PRP treated with abciximab) PRP. ScFv +ve and scFv -ve capsules were individually subjected to these PRP groups and incubated for 20 min. **Figures 3**a and S11a show specific targeting of scFv +ve capsules to the activated PRP, shown by the higher fluorescence intensity compared to other PRP groups (i.e., non-activated and blocked). No fluorescence intensity shifts were observed in the scFv -ve variants (Figures 3a and S11b).

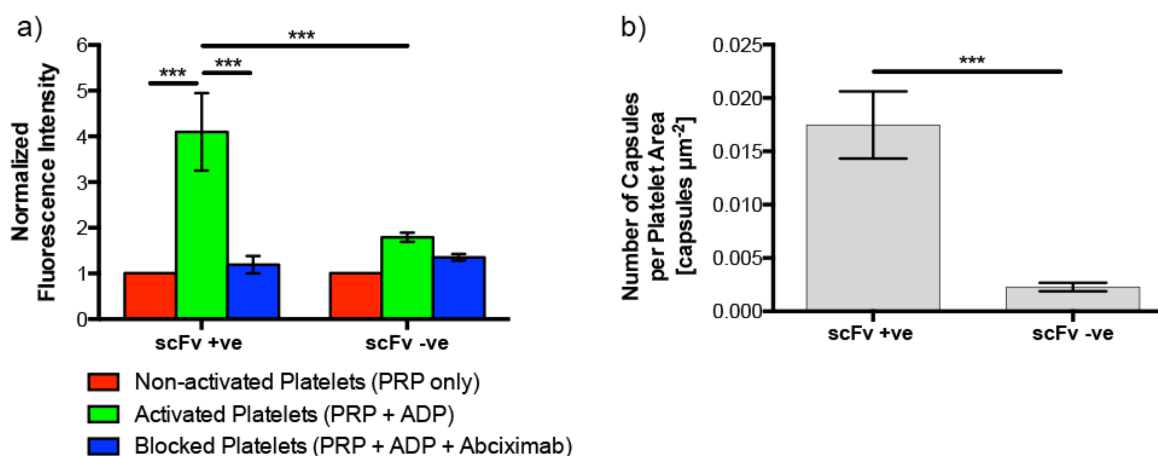


Figure 3. Flow cytometry analysis of the scFv affinity toward surface-bound GPIIb/IIIa receptors on the activated platelet surfaces. ScFv (+ve or -ve)-functionalized polymer capsules were targeted to: (a) three groups of platelet-rich plasma (PRP): non-activated platelets (PRP only, red), activated platelets (PRP + ADP, green), and blocked platelets (PRP + ADP + abciximab, blue) in static (non-flow) setting (mean ± SEM, n = 5, *** $p<0.001$); (b) activated platelets in a flow chamber setting (mean ± SEM, n = 9, *** $p<0.0001$).

Flow chamber experiments were performed in microfluidic flow channels that contained adhered activated platelet spreads. ScFv +ve and scFv -ve capsules were individually introduced at 110 s^{-1} (low venous shear rate)[22] for 1 min. Figures 3b and S12 show specific targeting of scFv +ve capsules toward activated platelets. In brief, the specific targeting behavior of scFv +ve capsules toward surface-bound GPIIb/IIIa receptors expressed on the activated platelets was shown.

To examine the influence of scFv functionalization on degradation kinetics, six sets of capsules (degradable or non-degradable, which were non-functionalized, scFv +ve or scFv -ve functionalized) were subjected to thrombin (2.5 units mL^{-1}).[20b,20c] Degradation profiles were found to be similar (complete degradation after 1.5 h), regardless of their surface chemistry (Figure S13). This suggests that scFv-functionalization of capsules did not influence thrombin sensitivity.

In conclusion, we prepared multifunctional polymer capsules based on a B-PEtOx$_{Alk}$ polymer via LbL assembly on MS particle templates. An active thrombolytic agent, uPA, was successfully encapsulated. The incorporation of a thrombin-sensitive cross-linker yielded stable polymer capsules. Exposure to thrombin showed concentration-dependent degradation and release kinetics. Surface-functionalization with a scFv antibody that has a high affinity toward the abundant GPIIb/IIIa integrin resulted in specific targeting to activated platelets. These multifunctional polymer capsules have been designed to be inherently responsive to thrombus microenvironments and represent a platform for developing a carrier system to induce thrombolysis at the area of acute thrombosis. This design concept is an important step

for developing intelligent systems that exploit inherent biological conditions to activate therapeutic action and provides an avenue for advanced therapeutic delivery systems.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Target and Act: Smart poly(oxazoline) (POx)-based multifunctional polymer capsules, which specifically target glycoprotein (GP) IIb/IIIa on the surface of activated platelets, are degraded by the serine protease thrombin and release the urokinase plasminogen activator loaded into the polymer capsules, only in the area of activated platelets.

Keywords antibody, drug delivery, plasminogen activators, polymer carriers, thrombosis

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Multifunctional Thrombin-Activatable Polymer Capsules for Specific Targeting to Activated Platelets