Enzyme Catalysis

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Bio-Click Chemistry: Enzymatic Functionalization of PEGylated Capsules for Targeting Applications**

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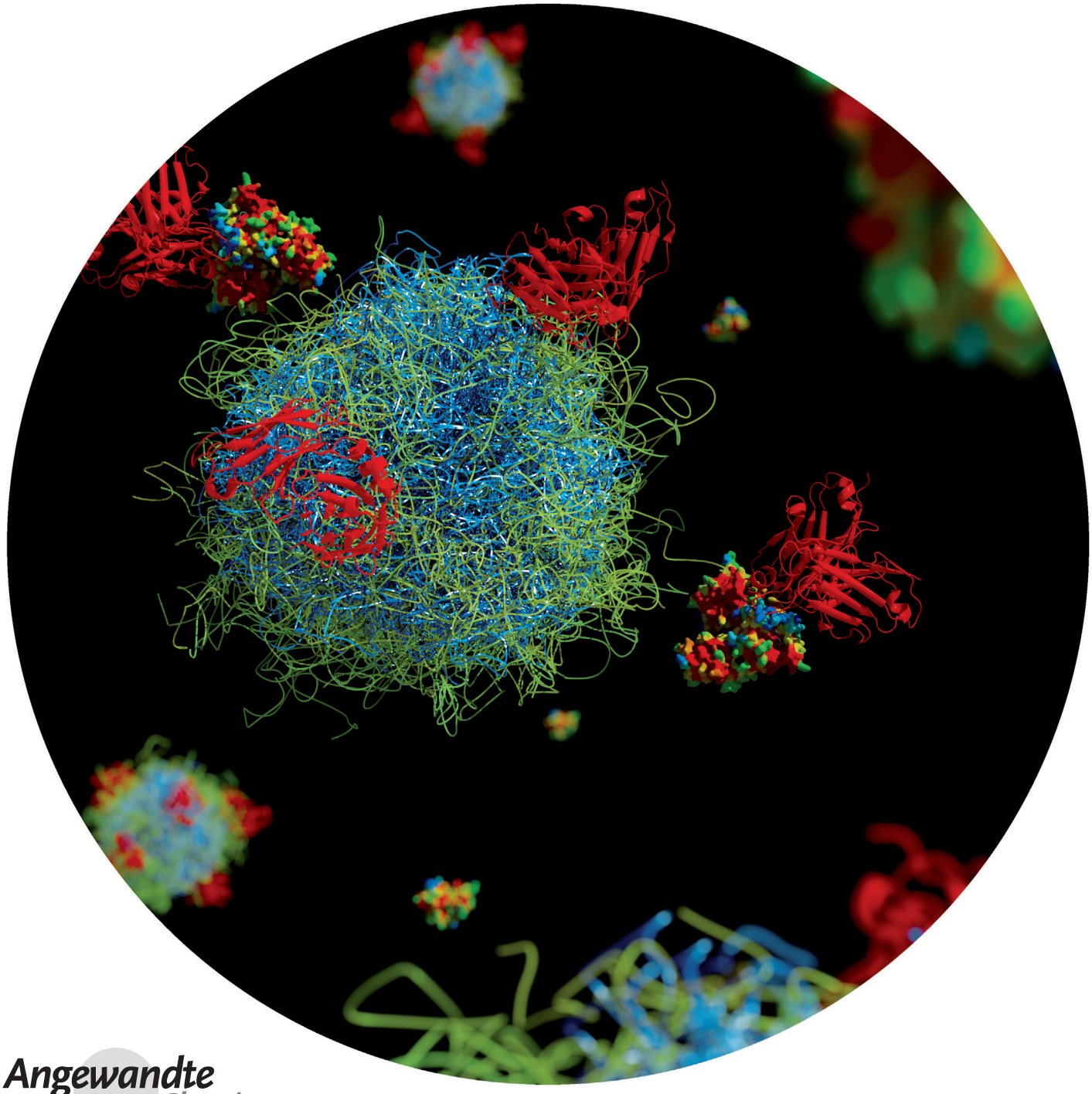

Targeted delivery with nanoengineered biofunctionalized pharmaceutical carriers has the potential to maximize the uptake of drugs at a diseased site while minimizing therapeutic side effects.[1] Effective drug delivery can be significantly enhanced through covalent and site-specific protein immobilization on the carrier vehicles, as this approach avoids random orientation and inactivation of cell-targeting ligands/antibodies. Although covalent biofunctionalization can be readily performed on protein functional groups using carbodiimide- and maleimide-based methods, these approaches do not provide site-directed attachment owing to the abundance of protein functional groups. Advanced techniques such as click chemistry have been commonly used for site-specific immobilization;[2] however, this method requires the incorporation of synthetic functional groups into biomolecules. More recently it has been demonstrated that antibodies expressed as fusion proteins with a SNAP tag (O_6-alkylguanine-DNA alkyltransferase)[3a] can be coupled to O_6-alkylguanine modified particles.[3b] Other enzyme-based coupling approaches have also been reported for site-selective conjugation but are limited due to the large size of the fusion proteins and the multiple preparation steps.[4] Hence, there remains a need for an efficient method for the covalent and site-specific functionalization of drug carriers.

A relatively new method for bioconjugation is based on the *Staphylococcus aureus* enzyme Sortase A (Srt A), which covalently attaches proteins to the bacterial cell wall.[5] Srt A recognizes protein substrates that contain an LPETG peptide motif and cleaves between the threonine and glycine residues; the carboxyl group of threonine subsequently undergoes a nucleophilic attack by the N-terminal amino group of a polyglycine, thus forming an amide bond.[6] Since its discovery, Srt A has been used to synthesize peptide conjugates with sugars,[7] glycolipids,[8] and peptide nucleic acids;[9] these conjugates are typically difficult to obtain by chemical reactions. The application of Srt A has also been extended to in vitro and in vivo studies for labeling proteins on living cell surfaces and for molecular imaging.[10] Recently, this enzyme has been employed to immobilize proteins on solid supports;[11] however, these studies have not been aimed at the development of therapeutic delivery systems for targeting applications. Herein, we report the use of Srt A as a "bio-click" approach for the functionalization of polymer drug vehicles. This enzymatic method possesses many of the useful properties of click chemistry; namely, it is highly specific, efficient, proceeds under mild conditions, and has no unwanted side reactions.

A phage-display-derived single-chain variable fragment (scFv) was used as a model antibody to demonstrate the Srt A catalyzed functionalization of polymer capsules and their subsequent targeting applications. This class of antibody fragment consists only of the variable regions of immunoglobulins and has the same targeting specificity of the parental form. Nonetheless, owing to their small size, scFvs have low immunogenicity with more-efficient tissue penetration, and thus have advantages over whole antibodies for targeting applications.[12] Moreover, genetic engineering allows easy introduction of functional residues into scFvs, making them suitable for site-specific coupling. The scFv used in this work has been shown to bind selectively to the activated glycoprotein (GP) IIb/IIIa receptor on platelets,[13] which in turn aggregate via GPIIb/IIIa to form thrombi. As platelet aggregation plays a critical role in thrombosis, GPIIb/IIIa presents a therapeutic target for the disease.[13] To make this scFv a suitable substrate for Srt A, it was encoded with an LPETG sequence (scFv-LPETG) at the C terminus, which is not within the targeting regions of the scFv, thereby preserving its bioactivity. In addition, to aid purification this scFv was also cloned with a histidine 6 (H_6) tag after the LPETG motif; this tag is later cleaved during the enzymatic ligation. Hence, Srt A mediates both the coupling reaction and the removal of the purification tag in a single step.

To develop scFv-functionalized delivery vehicles, low-fouling capsules assembled by the layer-by-layer (LbL) technique were used as the carrier system. Low-fouling materials such as poly(ethylene glycol) (PEG) and poly(*N*-vinyl pyrrolidone) (PVPON) are of interest for drug delivery as they exhibit a low level of nonspecific binding to cells. The capsules were assembled through hydrogen bonding by alternately layering alkyne-modified (1%) PVPON ($PVPON_{Alk}$)[14] and poly(methacrylic acid) (PMA) on silica particles ($\approx 3\,\mu m$ diameter), followed by deposition of alkyne-modified (5%) PEG (PEG_{Alk})[15] for PEGylation (Figure 1). The alkyne groups of the multilayer film were then cross-linked with a bisazide linker[14] using alkyne–azide click chemistry. To enable the immobilization of scFv-LPETG on the PEG_{Alk} surface, using click chemistry, the particles were functionalized with an azide-modified triglycine-containing peptide. This peptide (GGGWWSSK) had the C terminal lysine residue modified with 4 PEG units that were terminated with an azide group. Subsequently, the GGG-PEG-Az-modified particles were capped with sacrificial hydrogen-bonded PVPON/PMA films to impart colloidal stability to the particles upon core dissolution in hydrofluoric acid (HF). It was found that without the capping layers, the peptide-coated capsules aggregated in the presence of HF

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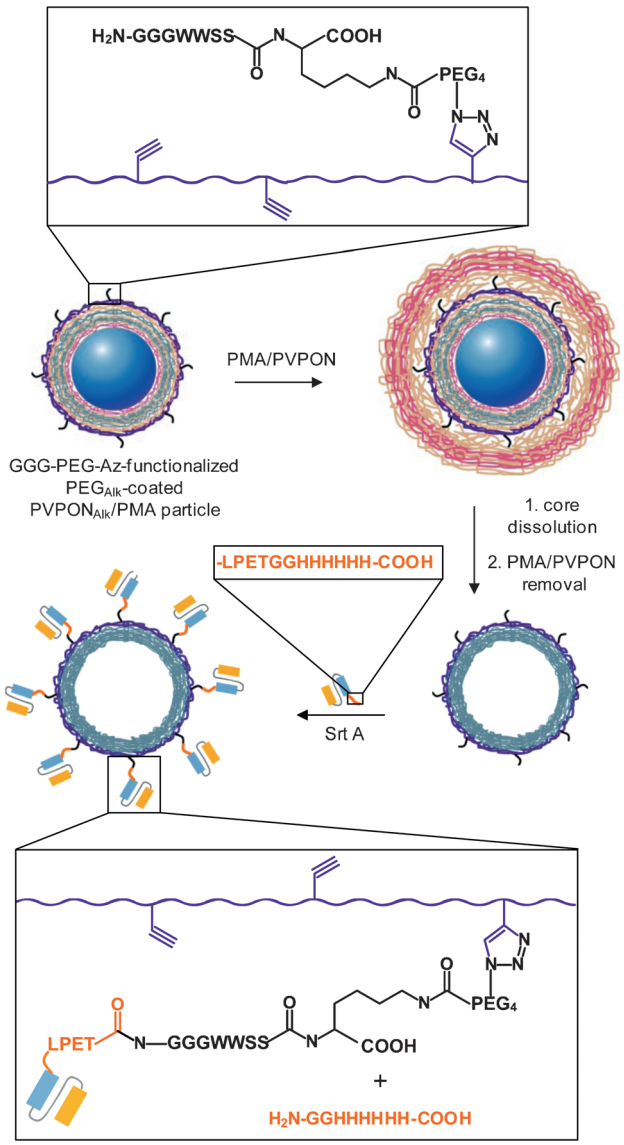

Figure 1. Preparation of scFv-functionalized PEGylated $PVPON_{Alk}$ capsules using Srt A. $PVPON_{Alk}$/PMA LbL particles terminated with PEG_{Alk} were functionalized with GGG-PEG-Az, followed by adsorption of protective capping layers of PMA/PVPON. The silica templates were then dissolved in HF and the resulting capsules were exposed to pH 7 to release PMA and PVPON. The GGG-PEG-Az-coated $PVPON_{Alk}$/PEG_{Alk} capsules were finally incubated with scFv-LPETG in the presence of Srt A, thus yielding scFv-functionalized capsules (in which the scFv purification tag was removed).

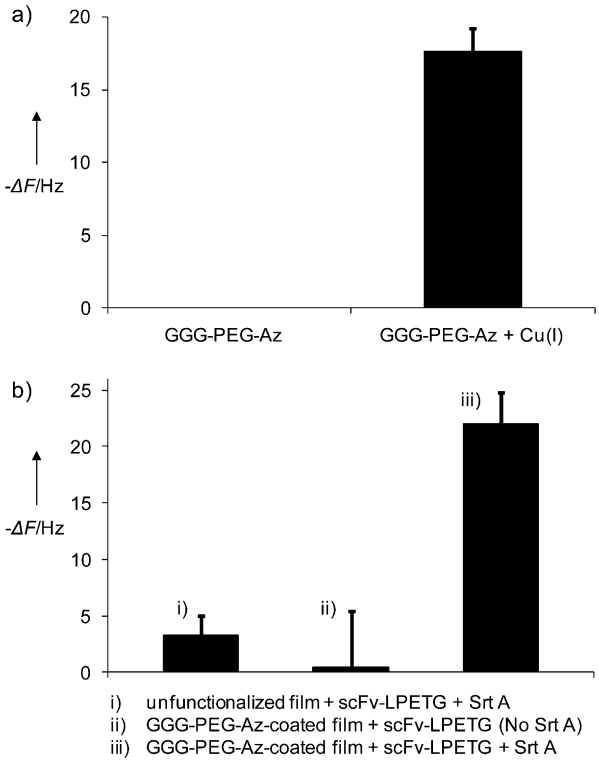

Figure 2. Frequency change (ΔF) for the functionalization of PEG_{Alk}-terminating films with GGG-PEG-Az and scFv-LPETG, monitored by QCM. a) PEG_{Alk}-terminating films were modified with GGG-PEG-Az in the absence and presence of Cu^{I} by click chemistry for 30 min in pH 5 sodium acetate (150 mM). b) scFv-LPETG was immobilized on i) the unfunctionalized films in the presence of Srt A, and on the GGG-PEG-Az-coated films in the absence (ii) and presence (iii) of Srt A. The films were incubated with 0.1 $g\,L^{-1}$ of Srt A and scFv-LPETG at 37°C for 1 h at pH 8 (50 mM tris(hydroxymethyl)aminomethane (Tris), 150 mM sodium chloride).

(data not shown). After removal of the core, the capsules were exposed to pH 7 to remove PVPON and PMA by disrupting the hydrogen bonds, thus forming GGG-PEG-Az-modified $PVPON_{Alk}$/PEG_{Alk} capsules. Finally, scFv-LPETG was conjugated to the capsules in the presence of Srt A to yield scFv-functionalized PEGylated capsules (See Supporting Information for experimental details.)

The immobilization of scFv-LPETG on multilayer films was first monitored on planar supports by using quartz crystal microgravimetry (QCM; Figure 2). Preliminary studies were conducted on a PEG_{Alk}-terminating film, as a model system, where PEG_{Alk} was hydrogen bonded to a poly(ethylenimine) (PEI)/PMA_{Alk} coated silicon dioxide sensor and the layers were cross-linked with the bisazide linker. GGG-PEG-Az was then covalently coupled to the PEG_{Alk} surface by the Cu^{I}-catalyzed cycloaddition reaction. In these studies, the ratio of dissipation to frequency was high ($> 1 \times 10^{6}$ per 10 Hz); thus the Sauerbrey equation is not valid.[16] However, the dissipation/frequency ratio remained similar throughout the experiments, hence a change in frequency is indicative of a change in mass. In the presence of Cu^{I}, a frequency change of −17.7 Hz was obtained for the attachment of GGG-PEG-Az while there was no significant frequency change in the absence of the catalyst (Figure 2a). This indicates the critical role of Cu^{I} as the catalyst to promote the covalent linkages of GGG-PEG-Az to the PEG_{Alk} surface, thus making the films a suitable substrate for the subsequent enzymatic coupling.

Next, the GGG-PEG-Az-coated and GGG-PEG-Az-uncoated films were functionalized with scFv-LPETG in a pH 8 buffer solution at 37°C. After 1 hour incubation, there was no further change in frequency and the films were washed with the buffer to remove any unbound scFv and the frequency change was measured. The Srt A catalyzed coupling of scFv-LPETG resulted in a frequency change of

−22.0 Hz on the GGG-PEG-Az-modified film (Figure 2b, iii). In the absence of Srt A, no significant frequency change was seen (−0.5 Hz) on the triglycine-coated surface (Figure 2b, ii). When the scFv was deposited on the unfunctionalized film, the adsorption was low (−3.3 Hz), even in the presence of the enzyme (Figure 2b, i). These studies confirm that the conjugation of scFv-LPETG to the GGG-PEG-Az-modified films was specifically catalyzed by Srt A. Furthermore, the low level of nonspecific binding of the scFv to the functionalized/unfunctionalized surfaces through physical adsorption revealed the low-fouling properties of the PEGylated films. This Srt A mediated immobilization technique provides a platform for fast (< 1 h), covalent, and site-specific functionalization of polymer films with biomolecules under mild conditions (pH 8).

The immobilization of scFv-LPETG was also performed on capsules under the conditions investigated above. Functionalization of $PVPON_{Alk}/PEG_{Alk}$ capsules with the scFv was verified using fluorescently labeled scFv (Figure 3) and the

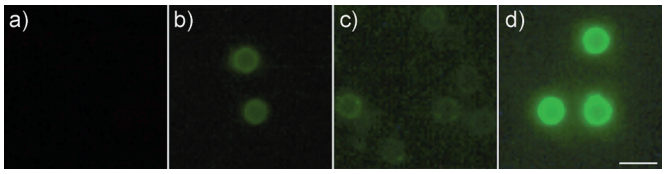

Figure 3. Fluorescence microscopy images of AF488-labeled scFv-LPETG coupled to $PVPON_{Alk}/PEG_{Alk}$ capsules. a) Unfunctionalized capsules, b) capsules lacking GGG-PEG-Az incubated with scFv-LPETG and sortase, c) capsules with GGG-PEG-Az incubated with scFv-LPETG but without sortase, d) capsules with GGG-PEG-Az incubated with scFv-LPETG and sortase. Scale bar = 5 μm.

amount of conjugated scFv was quantified using fluorescence spectroscopy. It was determined that approximately 0.36 μg of protein was coupled to 2.6×10^6 capsules, corresponding to 3×10^6 scFv per capsule. This finding suggests that the scFv is densely packed on the surface of the capsules, and is in agreement with our previous study on the antibody functionalization of capsules using click chemistry.[2a] Although copper-catalyzed azide–alkyne cycloaddition (CuAAC) and sortase-mediated functionalization both afford similar coupling densities, each method offers its own advantages. CuAAC coupling is a convenient method for attaching whole antibodies to surfaces, whereas sortase-mediated functionalization is well suited for coupling engineered recombinant proteins, such as scFv or modified antibodies, to surfaces with a controlled and defined orientation.

To evaluate the targeting ability of the enzymatically functionalized polymer carriers, the binding of scFv-functionalized capsules to activated GPIIb/IIIa on thrombi was examined. The specificity of the GPIIb/IIIa-specific scFv (scFv(+)) was compared to a mutated scFv (scFv(−)), which was also cloned with an LPETG tag. GGG-PEG-Az-modified $PVPON_{Alk}/PEG_{Alk}$ capsules were functionalized in the presence of Srt A with the two scFvs to form scFv(+)- and scFv(−)-coated capsules. Unfunctionalized $PVPON_{Alk}/PEG_{Alk}$ capsules were used as a control to investigate the background binding of the polymer capsules. Thrombi were formed from human platelet rich plasma and incubated with the three types of functionalized and unfunctionalized PVPON/PEG capsules (30 min, 37 °C). To analyze the binding, near-infrared imaging was used for visualization of the capsule-bound thrombi and the capsules were therefore labeled with DyLight 800 ($DL800_{Az}$). After incubation, the thrombi were washed in phosphate-buffered saline (PBS) to remove unattached capsules and the number of bound capsules was expressed qualitatively as fluorescence intensity (Figure 4). The scFv(+)-coated capsules demonstrated a high

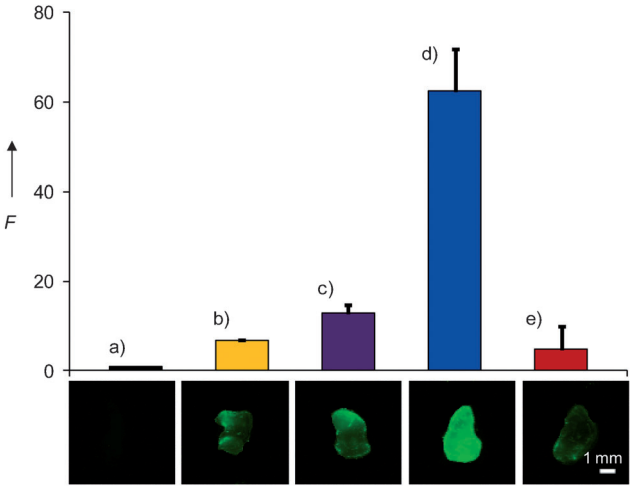

Figure 4. Targeting of scFv-functionalized capsules to thrombi. Relative fluorescence intensities (*F*) and images of (from left to right) a) untreated and DL800-labeled b) unfunctionalized, c) scFv(−)-functionalized, and d) scFv(+)-functionalized $PVPON_{Alk}/PEG_{Alk}$ capsule-bound thrombi, and e) a scFv(+)-blocked thrombus attached with scFv(+)-coated capsules. Targeting was conducted for 30 min at 37 °C in pH 7.2 PBS (with Ca/Mg) and analyzed by near-infrared imaging. The fluorescence intensity of untreated thrombi was set at 1.

level of specific binding to the thrombi with a high fluorescence intensity of 62.5 (the relative fluorescence intensity of untreated thrombi was set at 1). This represents a nine- and five-fold increase in binding compared to the scFv(−)-coated and uncoated capsules, respectively, thus indicating the targeting ability of the scFv(+)-coated capsules. Moreover, low levels of nonspecific binding of the control capsules were exhibited, thus illustrating the low-fouling properties of the PEGylated capsules. This finding is in accordance with other studies with PEGylated capsules.[17] The selective binding of the scFv(+)-coated capsules was also examined by a competitive-binding assay. The thrombi were first incubated with excess free scFv(+) to block the GPIIb/IIIa receptors before exposure to the scFv(+)-coated capsules. Targeting of the scFv(+)-coated capsules to blocked thrombi showed a 13-fold decrease in binding, thus confirming that the targeting of the scFv(+)-coated capsules to thrombi is highly specific and is GPIIb/IIIa mediated.

In summary, we have demonstrated an efficient method for the covalent and site-specific protein functionalization of polymer capsules using Srt A. Owing to the precision of the

ligation of genetically encoded substrates, this bio-click approach allows targeting ligands to be oriented with the antigen-binding sites available for binding, thus retaining their bioactivity. The GPIIb/IIIa-specific scFv-functionalized capsules showed a high level of specific targeting to thrombi, thus making them promising for anti-thrombotic and thrombolytic therapy. Hence, we expect this Srt A mediated biofunctionalization technique to find a range of applications in functionalizing materials for use in drug delivery as well as diagnostics and imaging.

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