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Particle generation, functionalization and sortase A-mediated modification with targeting of single-chain antibodies for diagnostic and therapeutic use

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Surface Modification of Nanoparticles with Triglycine Peptides for Sortase A-mediated ScFv Conjugation

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Abstract

Antibodies are a rapidly growing class of human therapeutics and the second largest group of drugs after vaccines. Antibody fusion to non-protein material such as contrast agents / radiotracers, nanoparticles or small molecule drugs is currently of great interest for molecular imaging and drug delivery. Progress in the field is still limited by non-directed bioconjugation techniques impairing antibody affinity, reducing product yields and creating multicomponent mixtures. We present a detailed protocol for enzymatic bioconjugation between a small recombinant single-chain variable fragment (scFv) and imaging particles, as well as conjugation to drug delivery nanocapsules. Site-specific bioconjugation is even more important for small recombinant antibodies such as scFvs compared to full sized IgG antibodies as the chance of negatively impacting on antigen binding increases with reduced antibody size. The scFv is equipped with a short C-terminal LPETG tag and the fusion partners carry a N-terminal GGG nucleophilic group. Both parts are then conjugated in the presence of the transpeptidase Sortase A from *Staphylococcus aureus*. This method provides an efficient conjugation approach, and importantly leaves the functionality of the antibody intact.

Introduction

Antibody conjugation to imaging agents, drugs, nanoparticles and drug delivery vehicles has the potential to significantly increase the utility of existing and new antibodies{Kim:2010bw}. However, most antibody conjugation methods still target endogenous amino acids, such as the amino group of lysine or the thiol group of cysteine commonly distributed throughout the antibody structure{Hermanson:2008ui}. When the conjugation involves critical residues essential for antigen binding, the functionality and affinity of the antibodies, particularly small recombinant antibody fragments, will be impaired.

Site-specific bioconjugation is now widely recognized as a key technique in modern biotechnology. The ability to label antibodies in a site-specific and selective manner can minimize any adverse effects on the antibodies' functionality. A single covalent bond between the antibody scaffold and conjugation partner, preserving the functionality of both components, is of critical importance. Achieving this goal in an effective way has a multitude of applications in diagnostics as well as in therapeutic antibody development. Sortase A from *Staphylococcus aureus* is an emerging bioconjugation tool{Popp:2011er, Popp:2009ic,

Popp:2007hp}, but its suitability for antibody conjugation has only recently been investigated{Ta:2011eq, Leung:2012hz, Wang:2012ef, Madej:2011ic, Witte:2012ec}.

Sortase A bioconjugation

The Sortase A transpeptidase enzyme consists of 206 amino acids (17.9 kD){Schneewind:1992wx}. In its physiological environment the enzyme links surface proteins of *S. aureus* to the bacterial cell wall{Marraffini:2005jr}. The transpeptidase domain catalyzes the cleavage of a short 5 amino acid recognition sequence (LPXTG) (X being any amino acid) with the concomitant formation of a peptide bond between a N-terminal (oligo)glycine peptide as the nucleophile and the target protein{Popp:2009ic}. After recognition of the Sortase LPXTG motif, the catalytic cysteine residue in the enzymatic site serves as a nucleophile to cleave the peptide bond between threonine and glycine. Cleavage occurs via a thioacyl intermediate, which is resolved by attack of the N-terminus of an (oligo)glycine nucleophile, thereby creating a new stable peptide bond that links the substrate to the incoming nucleophile (schematically shown in Fig. 1). Successful transpeptidation can be achieved with nucleophiles containing one to five glycines; however, maximum reaction rates are obtained when two or more glycines are present{Chan:2007dc, Popp:2009ic}. Sortase A mediated bioconjugation has many advantages: it is highly specific, proceeds under mild conditions and in the presence of excess incoming nucleophile has little to no unwanted side reactions. Sortase A conjugation can be readily adapted to produce a wide range of compositions with different features, including drug delivery vehicles{Leung:2012hz} or molecular imaging agents{Ta:2011eq, Wang:2012ef}.

Conjugation partners for molecular imaging and drug delivery

The following protocol describes the step-by-step procedure of Sortase A mediated bioconjugation between recombinant scFv and

- 1) Particles of iron oxide for magnetic resonance imaging{Ta:2011eq},
- 2) Layer-by-layer capsules for drug delivery{Leung:2012hz} and

The conjugation method comprises of 3 stages:

- (1) expression and purification of the scFv-LPETG against activated platelets, a non-binding control scFv_{mut}-LPETG as well as the transpeptidase Sortase A,
- (2) preparation of GGG-coated conjugation partners as nucleophiles for the enzymatic reaction and
- (3) incubation of triglycine-tagged components with Sortase A and scFv-LPETG.

The antibody used for our studies targets the glycoprotein (GP) IIb/IIIa ($\alpha_{IIb}\beta_3$, CD41/CD61) receptor, the most abundant molecule on the platelet surface{Hagemeyer:2010wm}. This integrin receptor undergoes a conformational change upon platelet activation, exposing epitopes that are uniquely specific for activated platelets{Armstrong:2012cu}. Activated platelets are critical players in atherosclerosis, thrombosis, and inflammation{Gawaz:2005ks, May:2008cj} and are therefore of high interest in the fields of molecular imaging and drug delivery. We used three variants of the antibody: a non-blocking anti-LIBS (ligand-induced binding sites) antibody{Stoll:2007jy, Schwarz:2004go} for our imaging application, a function blocking antibody SCE5{Schwarz:2006eb, Schwarz:2004iw} for the drug delivery applications and a non-binding antibody as control for all experiments. To make the scFvs suitable for the bioconjugation reaction, a Sortase A LPETG recognition tag was added to the C-terminus N-terminal to a tag containing 6 histidine, used for purification and detection, thereby creating scFv-LPETGGGH₆. This C-terminal part of the antibody scaffold is not utilized in antigen binding, thus maintaining antibody functionality. In addition, by placing the LPETG motif before the His₆-tag, this part is cleaved off during the enzymatic reaction facilitating both coupling and removal of the purification tag in one step.

This protocol has been used successfully to conjugate scFv to GGG modified commercial iron oxide microparticles (MPIOs) for magnetic resonance imaging. In addition, we have established a detailed protocol for the development of scFv-functionalized low-fouling nanocapsules assembled by the layer-by-layer (LbL) technique as drug delivery vehicles. The scFv was conjugated to the nanocapsules in the presence of Sortase A, yielding scFv-functionalized PEGylated capsules. The underlying principle is the introduction of a small GGG peptide, which can be used as a nucleophile in the subsequent Sortase A conjugation. This can either be achieved by “click chemistry” of an azide carrying GGG peptide conjugated to surface alkyne groups of LbL assembled capsules or peptide bond generation between surface amines and a protected GGG peptide.

Advantages and limitation of the Sortase approach

Several other enzymatic conjugation techniques have been developed, the most prominent being the Biotin ligase - AviTag enzyme technology. This approach takes advantage of the strong binding between biotin and (strept)avidin but avoids possible inactivation of proteins of interest by random biotinylation associated with chemical labeling. The AviTag sequence with only 15 amino acids (GLNDIFEAQKIEWHE) contains the optimal peptide sequence reported to be biotinylated by BirA{Beckett:1999ff}. AviTag can be cloned into either the N-

terminal{Chen:2005jt} or C-terminal{Sung:2011bo} of a fusion protein, or at other internal protein locations{Tannous:2006ge}, as long as the peptide domain forms a surface-exposed accessible loop. This is one advantage over the Sortase technique, which is restricted to the C-terminus of the antibody or targeting protein. Although the AviTag is not much larger than the Sortase tag, the conjugation partner, (strept)avidin, is significantly larger than the GGG motif utilized by Sortase. This large size (56-69 kDa) can affect protein function{Howarth:2008dt}. Furthermore, (strept)avidin is potentially immunogenic and may bind to endogenous biotin. Although ketone-modified biotin molecules have recently been developed (allowing conjugation of ketone-tagged proteins with hydrazide- or hydroxylamine-functionalized molecules{Chen:2005jt}) this approach lacks sensitivity due to the specific kinetics of hydrazide labeling.

Another enzymatic conjugation technique is the transglutaminase catalyzed formation of peptide bonds between the γ -carboxamide group of an intact protein-bound glutamine and a variety of primary amines, particularly the ϵ -amino group of lysine{Lin:2006ew}. Similar to Sortase, the recognition sequences (Q-tags) such as PKPQQFM{Taki:2004fu} and GQQQLG{Hu:2003cf} are small and very specific and can be genetically introduced at the N-terminus of the target proteins.

Microbial transglutaminase from *Streptomyces mobaraensis* has already been used for labeling of scFvs{Takazawa:2004bf} and for site-specific modification and PEGylation of different pharmaceutical proteins for clinical applications{Besheer:2011jm}. With its high selectivity, mild reaction conditions and highly stable covalent bond, transglutaminase conjugation has many attractive features comparable to Sortase. However in the absence of the canonical Q-tag, labeling of non-Q-tagged proteins{Lin:2006ew} may occur whereas Sortase is absolutely specific for the LPXTG tag.

Compared to other techniques, the primary advantage of Sortase bioconjugation is the requirement of two very short amino acid motifs (LPXTG and GGG) that are easily incorporated into substrates of interest by either chemical synthesis or genetic engineering. This minimizes issues associated with protein expression and purification as well as avoiding adverse immunogenic complications, one of the major translational hurdles of the AviTag system. In addition, the high specificity of Sortase allows coupling of crude protein preparations, thereby shortening protein purification protocols while preventing ligation of contaminants{TonThat:1999ur}. This lack of specificity is another main disadvantage of the transglutaminase technology. Furthermore, Sortase is easy to produce in large amounts, making this method cost efficient{Clow:2008df}.

One of the major drawbacks of the Sortase approach is the slow reaction kinetics, ranging from 1-3 h to (in some cases) overnight incubation periods to achieve a high conjugation yield{Clow:2008df}. However, new exciting work using mutagenesis and maturation studies indicates that Sortase A activity can be improved up to 140 times using a directed evolution strategy involving yeast display{Chen:2011er}. Another drawback is the difficulty in controlling the amount of coupled proteins, as the product still displays the LPXTG Sortase recognition sequence{Wu:2010ko}. This reverse reaction leads to constant turnover of formed product. In addition, due to the nature of the Sortase reaction, introduction of LPXTG- and GGG-motifs to the target is restricted to the C-terminus and N-terminus, respectively.

Overall, this protocol demonstrates the novel use of Sortase A for bioconjugation of recombinant single-chain variable fragment in a selective and site-specific manner, which allows the production of targeted imaging contrast particles, drug delivery vehicles suitable for molecular imaging and targeted drug therapy. The Sortase A reaction is universally applicable and adding Sortase to the toolbox for recombinant antibody modification has the potential for significant impact.

MATERIALS

Reagents

- 1 kb DNA ladder (New England Biolabs, cat. no.N3232S)
- ACK lysis buffer (GIBCO, Invitrogen, cat. no. A10492-01)
- Acetic acid (Sigma-Aldrich, cat. no. 320099)
- Acrylamide (acrylamide-Bis (37.5:1), 30% (wt/vol) aqueous solution, VWR, cat. no. 1.00639.1000)
- Agarose (Sigma-Aldrich, cat. no. A9539)
- Anti-6x His tag horseradish peroxidase (Abcam cat. no. Ab1187)
- Host optimized DNA from Geneart/Invitrogen with C-terminal LPETG tag N-terminal of a His₆-tag for the scFvs anti-LIBS {Stoll:2007jy, Schwarz:2004go}, SCE5 and mutMA2 {Schwarz:2006eb, Schwarz:2004iw}.
- Ampicillin (Sigma-Aldrich, cat. no. A0166)
- APS (ammonium persulfate, Sigma-Aldrich, cat. no. A3678)
- BL21Star™ (DE3) (Invitrogen cat. no. C6020-03)
- BOC-HN-linker-NH₂ peptide (GL Biochem Ltd, Shanghai, China)
- BOC-HN-GGGWW-COOH peptide, 734 Da (GL Biochem Ltd, Shanghai, China)
- BugBuster® Master Mix (Merckmillipore cat. no. 71456)
- BSA (Bovine serum albumin, Sigma-Aldrich, cat. no. A7030)
- CaCl₂ (calcium chloride, Sigma-Aldrich, 223506)
- DCM (dichloromethane, Sigma-Aldrich, cat. no. 270997)
- C₆H₇NaO₆ (sodium ascorbate, Sigma-Aldrich, cat. no. A7631)
- C₂H₃NaO₂ (sodium acetate, Sigma-Aldrich, cat. no. S2889)
- C₆H₅Na₃O₇ x 2 H₂O (sodium citrate tribasic dehydrate, Sigma-Aldrich, cat. no. C8532)
- CuSO₄ (copper sulfate, Sigma-Aldrich, cat. no. 451657)
- DIPEA (N,N-diisopropylethylamine, Sigma-Aldrich, cat. no. 496219)
- DMSO (dimethyl sulfoxide, Sigma-Aldrich, cat. no. D8418)
- Dynabeads M-270 Amine (Invitrogen, cat. no. 143-07D)
- EDTA (Sigma-Aldrich, cat. no. 431788)
- Gel Loading Dye, Blue (New England Biolabs, cat. no.B7021)
- Glycerol (Sigma-Aldrich, cat. no. G5516)
- LB Broth (Luria Bertani Broth, Sigma-Aldrich, cat. no. L3022)
- Na₂B₄O₇ (sodium borate, Sigma-Aldrich, cat. no. S9640)

- NaCl (sodium chloride, Amresco Inc, cat. No. 0241)
- NEAA (100x, Liquid, GIBCO/Invitrogen, cat. no. 11140-035)
- $(\text{NH}_4)_2\text{SO}_4$ (ammonium sulfate, Sigma-Aldrich, cat. no. A4418)
- NH_2 -GGGWSSK-PEG₄-N₃ peptide (Mimotopes, Melbourne, Australia)
- Ni-NTA Superflow Columns (Qiagen, cat. no. 30622)
- Nucleus free water (New England Biolabs, cat. no. E7327)
- pET-20b(+) vector (Novagen® cat. no. 69739)
- Poly(methacrylicacid) (PMA, 30 wt%, $M_w=15$ kDa, Polysciences, USA, cat. no. 21170-100)
- Poly(*N*-vinyl pyrrolidone) (PVPON, $M_w = 55$ kDa, Sigma-Aldrich, cat. no. PVP40)
- PyBOP (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate, Sigma-Aldrich, cat. no. 377848)
- QIAquick gel-extraction kit (Qiagen, cat. no. 28706)
- Qiagen Miniprep kit (Qiagen cat. No. 27106)
- Restriction enzyme NotI (New England Biolabs, cat. no.R109)
- Restriction enzyme XhoI (New England Biolabs, cat. no.R146)
- Scalpel blade no 10 (Pacific Laboratory products cat. no. SB10)
- SiO₂ particles (~3 μm diameter as a 5 wt% suspension, MicroParticles GmbH, Germany, cat. no. SiO2-F-2.5)
- SnakeSkin Dialysis Tubing, 10 MWCO (Thermo Scientific, cat. no. 88245)
- SOC-Media (Super Optimal broth with Catabolic repressor, Invitrogen, cat. no. 15544-034)
- Host optimized DNA from Geneart/Invitrogen encoding Sortase A enzyme (recombinant variant with 59 amino-acid N-terminal deletion)
- SYBR®-Safe DNA gel (Invitrogen cat. no. S33102)
- T4 buffer and T4 DNA Ligase (New England Biolabs, cat. no. M0202S)
- TEA (triethanolamine, Sigma-Aldrich, cat. no. T1377)
- TEMED (N,N,N',N'-tetramethylethylenediamine, Sigma-Aldrich, cat. no. T9281)
- TFA (trifluoroacetic, Sigma-Aldrich, cat. no. T6508)
- Tris-HCl (Tris(hydroxymethyl)aminomethane hydrochloride, Sigma-Aldrich, cat. no. T6066)
- Trizma® base (Sigma-Aldrich, cat. no. T1503)
- 0.05% wt/vol Trypsin/EDTA (1X), Phenol Red (GIBCO/Invitrogen, cat. no. 25300-054)

- Tween 20 (Polysorbate 20, Sigma-Aldrich, cat. no. P2287)
- Water (Milli-Q water purification system, 18.2 M Ω , UV sterilized; Millipore or equivalent)
- Triethylene glycol 99% - Sigma-Aldrich T59455-25G
- Sodium Hydride (60% dispersion in mineral oil) – Aldrich 452912 -5G
- Propargyl bromide - 80% in toluene Aldrich 81831
- Sodium hydrogen carbonate – Sigma-Aldrich S6014-1KG
- Magnesium sulfate anhydrous – Sigma-Aldrich M7506-500G
- Silver chloride 99.999% - Aldrich 204382-5G
- 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) – Fluka 33482-50mL-F
- Chloro-trimethylsilane 98% - Aldrich 92361-100 mL
- Hydrochloric acid – 37% Sigma-Aldrich 320331-2.5L
- Triethylamine – Sigma-Aldrich T0886-100mL
- Methacryoyl chloride 97% - Sigma-Aldrich 64120-250 mL
- Copper (I) chloride – Acros 208391000 -100G
- 2,2'- bipyridine 99% - Sigma-Aldrich D216305 – 25G
- Methyl-2-bromo-propionate 98% - Aldrich 302392-5G
- OEGMA – Aldrich 447943 -500 mL (both monomers purified before use by running through a column of neutral alumina to remove inhibitor)
- MEO₂MA – Aldrich 409529 -500 mL (as above)

Equipment setup

- Agar plates: 100 mm x 20 mm Corning® Petri dishes (Sigma, cat. no. CLS430591-500EA)
 - Agarose gel set up: Sub-Cell GT UV-Transparent Wide Mini-Gel Tray (cat. no. 170-4426EDU) and Fixed-Height Comb (cat. no. 170-4447EDU). All from Bio-rad.
 - Module (cat. no. 166-0827EDU). All from Bio-rad.
 - Centrifuge (Beckman Coulter, Allegra X-22R Series or equivalent)
 - Centrifuge (Eppendorf 5424 Series or equivalent)
 - 250 ml centrifuge bottles (Thermo Scientific, Nalgene* cat.no. 3120-0250)
 - Gel electrophoresis imager (BioRad Gel-Doc system with Quantity One software)
 - Direct Detect™ (Merck Millipore)
 - Direct Detect™ Spotting Tray (Merck Millipore, cat. no. DDMISC0010-ST)
 - Heat block (Stuart block heater, SBH130D or equivalent)
 - Clear plastic 15 ml and 50 ml centrifuge tube with graduations and a blue screw cap
 - Fridge (Fisher & Paykel or equivalent)
 - Filtered tips (Aerosol Barrier Pipette Tips or equivalent)
 - Magnetic Particle Concentrator (Dynal or equivalent)
 - Microbalance-XP2U Ultra (Mettler Toledo or equivalent)
 - Microscope (Olympus CKX41 or equivalent)
 - NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific)
- SDS-PAGE gel setup: Mini-PROTEAN Tetra Cell Casting Module (Bio-rad, cat.no. 165-8021EDU)
- Plastic trays for soaking gels
 - Rotary mixer (RSM7, Ratek or equivalent)
 - Plastibrand microcentrifuge tubes with cap 1.5ml (Eppendorf, Sigma-Aldrich or equivalent)
 - Thermomixer (Eppendorf or equivalent)
 - Vortex mixer (Eppendorf or equivalent)
 - Pipette boy Serological pipet (5ml, 10ml, 25ml)
 - NMR - ^1H NMR spectroscopy was performed using a Varian Unity 400 (400MHz) spectrometer with the deuterated solvent .
 - GPC - SEC was run at 50°C in DMF (flow rate 1mL/ min) using a Spectra Physics Instruments apparatus equipped with a UV-detector SEC-3010 and a refractive index detector
 - SEC 3010 from WGE Dr. Bures (Columns: Guard (7.5 x 75 mm), PolarGel-M (7.5 x 300 mm)), calibration with linear polystyrene standards.

REAGENT SETUP

Phosphate buffered saline (PBS) Dissolve 137 mM NaCl (8 g), 2.7 mM KCl (0.2 g), 10 mM of Na₂HPO₄ (1.44 g) and 2 mM KH₂PO₄ (0.24 g) in 800 ml distilled water. Adjust pH to 7.4. Adjust volume to 1 l with additional distilled water. Finally sterilize by autoclaving.

Ampicillin-supplemented LB-media Let LB-media cool down and then add ampicillin to 100 mg/ml.

Ampicillin-supplemented LB-agar LB-media, add 1.5% (w/v) agarose before autoclaving. Before use, add ampicillin to 100 mg/ml. Plates should be poured in advance and dried for approximately 1 h in a sterile cabinet to reduce condensation. LB-agar plates could be stored for a week in 4 °C. Before use, remove prepared plates from cold and incubate at 37 °C to avoid both condensation and shock to the cells.

LB-media autoclave LB-media.

50 % (v/v) Glycerol Dilute 100 % glycerol with same volume of Nucleus free water.

50 x TAE buffer 2 M Tris, 5 mM EDTA pH 8, 5.5% acetic acid.

1% (wt/vol) Agarose gel 100 ml of 1 x TAE buffer, 1 g agarose. Heat in the microwave until the agarose is completely dissolved and then add 1 µl of SYBR®-Safe DNA gel stain per 20 ml of gel.

Buffers used for 2.7 µm amine-tagged iron-oxide particles

Buffered hydrofluoric acid (HF) solution Prepare a solution of pH 5.0 buffered HF by mixing 100 µl of 8 M ammonium fluoride (29.63 mg / 100 µl) and 50 µl of 2 M hydrofluoric acid (2 mg / 50 µl).

! CAUTION Hydrofluoric acid and ammonium fluoride are very toxic. Extreme care should be taken when handling HF solution, and only small quantities should be prepared.

Washing buffer/PBS/Tween Add Tween-20 to PBS, pH 7.4 to a final concentration of 0.1% (v/v). Both can be stored for several months at room temperature.

Storing buffer Add Tween-20 to PBS, pH 7.4 to a final concentration of 0.01% (v/v). These buffers can be stored for several months at room temperature.

Sortase reaction buffer. Prepare 250 ml of sortase reaction buffer. Dissolve 50 mM Tris-HCl (1.5 g), 150 mM NaCl (2.19 g) and adjust the pH to 8.0. It can be stored for several months at room temperature.

Solutions for LbL capsules

Acetate buffer. Prepare 50 mM pH 4.0 acetate buffer and 150 mM pH 5.0 acetate buffer. It can be stored for several months at room temperature.

Make 1 mg/ml solutions of PMA (2 mg dissolved in 2 ml of 50 mM pH 4.0 acetate buffer), PVPON (0.5 mg in 0.5 ml of 50 mM pH 4.0 acetate buffer), PVPON_{Alk} (2 mg in 2 ml of 50

mM pH 4.0 acetate buffer) and PEG_{Alk} (0.5 mg in 0.5 ml of 150 mM pH 5.0 acetate buffer). Make solutions of bisazide crosslinker (0.5 mg in 500 μ l of 150 mM pH 5.0 acetate buffer), sodium ascorbate (1.3 mg in 300 μ l of 150 mM pH 5.0 acetate buffer), and copper sulfate (1.4 mg in 1 ml of 150 mM pH 5.0 acetate buffer). Make solutions of sodium ascorbate (1.3 mg in 300 μ l of 150 mM pH 5.0 acetate buffer) and copper sulfate (1.4 mg in 1 ml of 150 mM pH 5.0 acetate buffer) as well as azide functionalized Sortase peptide substrate (GGG-PEG-Az, 0.1 mg in 500 μ l of 150 mM pH 5.0 acetate buffer). Polymer and buffer solutions should be made fresh before the capsule assembly. Sodium acetate, copper sulphate and bisazide cross-linker solutions should be made fresh immediately before use.

PROCEDURES

Transformation of synthesized scFv-LPETG and Sortase construct into *E. Coli*

Timing 2-3 h +16 h (overnight)

* **NOTE** Single-chain antibodies with LPETG tag (Host optimized DNA) were synthesized by Geneart (Invitrogen). All constructs contained a C-terminal LPETG tag and a N-terminal His₆-tag.

* **NOTE** Sortase A enzyme, containing a N-terminal His₆-tag, was also synthesized by Geneart.

All constructs from Geneart with the required restriction sites were shipped in a standard shuttle vector in which the DNA is delivered as 5 μ g lyophilized plasmid DNA in microcentrifuge tube.

1. Centrifuge the microcentrifuge tubes at 10,000rpm on a bench top centrifuge (Eppendorf 5424) for 30 seconds.
2. Add 50 μ l of nucleus free water to the lyophilized plasmid DNA. The concentration of the DNA is 100 ng/ μ l.
3. Thaw NEB Turbo Competent *E. coli* cells on wet ice for 10 minutes.

▲ **CRITICAL STEP** Temperature and timing of thawing are important for outcome of transformation. Do not leave the cells standing for more than 30 minutes prior to use.

4. Add 100 ng of plasmid DNA onto thawed cells then carefully flick the tube 5 times.

! **CAUTION** Do not vortex.

5. Place cell mixture on wet ice for 30 minutes

! **CAUTION** Do not mix.

6. Heat shock cells for exactly 30 seconds at 42 °C

! **CAUTION** Do not mix.

❑ **CRITICAL STEP** Temperature and timing of the heat shock step are important and specific to the transformation volume and vessel.

7. Return to ice for 4 minutes

! **CAUTION** Do not mix.

8. Add 950 µl of room temperature SOC-Media onto the cells.

9. Place cells at 37°C for 60 minutes, shaking vigorously at 250rpm in a bacterial incubator.

10. Warm ampicillin-supplemented LB-agar plates to 37°C.

▲ **CRITICAL STEP** The resistance marker can vary between different vector systems therefore the resistance markers have to be carefully checked before adding suitable antibiotics.

11. Plate 100 µl of cells on to a LB-agar plate and grow overnight at 37°C in a bacterial incubator.

▲ **CRITICAL STEP** Keep your lab bench area sterile by working near a flame or Bunsen burner.

■ **PAUSE POINT** After overnight incubation, the plates should be sealed with parafilm and may be stored for up to 4 weeks in a cold room or fridge.

Growing of transformed *E.coli* in cultures, storage and DNA extraction.

● **TIMING** 14 hours

1. Pipette 5.5 ml of ampicillin-supplemented LB media into 50 ml clear plastic centrifuge tubes.

2. From the ampicillin-supplemented LB-agar plate, which has been growing overnight in 37 °C, inoculate the ampicillin-supplemented LB-media with single bacterial colonies using a plastic pipette tip.

▲ **CRITICAL STEP** Be careful when picking the bacterial colony, be careful not to contaminate with other colonies.

3. Place the plastic centrifuge tubes at 37°C for overnight (12 to 18 hours) in a bacterial incubator, shaking vigorously at a 30° angle.

* **NOTE** After incubation, check for growth, which is characterized by a cloudy haze in the media.

4. For long-term storage of bacteria, create a glycerol stock of cells. Add 500 µl of the overnight culture to 500 µL of 50% (v/v) glycerol in a 2 mL screw top tube or cryovial and gently mix.

* **NOTE** Shake the glycerol stock before freezing (5-6 times), making sure that it is one

uniform solution with no layers present. Warning: Snap top tubes are not recommended as they can open unexpectedly at -80°C .

5. Freeze the glycerol stock tube at -80°C .

▲ **CRITICAL STEP** Make sure that the glycerol stocks tubes are stored in -80°C . The stock is now stable for years; however subsequent freeze and thaw cycles reduce shelf life.

6. In order to recover bacteria from your glycerol stock for future use, open the tube and use a sterile loop or pipette tip to scrape some of the frozen bacteria off of the top.

* **NOTE** Do not allow the glycerol stock to thaw!

■ **PAUSE POINT** For future use, streak the bacteria on ampicillin-supplemented LB-agar plate and grow the plate overnight at 37°C . Start an overnight culture with a single colony for plasmid DNA prep the following day. A single colony from a freshly streaked agar plate to inoculate a bacterial culture for DNA purification is the best way to minimize the chance of having a mixture of plasmids in your purified DNA.

7. Pellet the cells by centrifuging the remaining 5 ml of bacterial culture at 4,000 rpm for 10 minutes. Remove the clear supernatant.

■ **PAUSE POINT** Bacterial pellets may be frozen at -20°C for several weeks.

8. Purify plasmid DNA using commercially available Qiagen Miniprep kit, following the manufacturer's instructions.

■ **PAUSE POINT** Elutions of the plasmid DNA from the Qiagen miniprep kit may be stored at -20°C for several months.

9. Measure the amount of DNA obtained from the preparations using NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific).

Molecular cloning of constructs into pET20(+) vector

Restriction digest of DNA

● **TIMING** 4-5 hours

1. Prepare a 20 μl restriction digest reaction for each DNA preparation comprising 10 μl of DNA, 2 μl of 10x buffer and 1 μl of NotI and 1 μl of XhoI. Incubate at 37°C for a minimum of 2 hours.

2. Add 4 μl of 6x gel loading dye to samples and load the samples on a 1% (w/v) agarose gel and 1 kb DNA ladder. Size-separation is performed using electrophoresis tank at 100V for 30 minutes.

3. Place the gel on a Bio-Rad gel electrophoresis imager and photograph.

Size selection of digested gel and purification of digested DNA.

- **TIMING** 2-3 hours

1. Determine the size of the digested construct (scFv-LPETG inserts are imaged near the 1kb mark, the Sortase A insert is imaged at the 1kb mark, and the digested vector pET20b(+) is imaged just above the 3 kb mark)
2. Use a clean scalpel blade and carefully cut around the area of the targeted size. Place the cut gel portion into labelled 1.5 ml microcentrifuge tubes.
3. Extract using a QIAquick gel-extraction kit, following the manufacturer's instructions.
 - **PAUSE POINT** Elutions of the plasmid DNA from the Qiagen gel-extraction kit may be stored at -20 °C for several months.
4. Measure the amount of DNA obtained from the preparations using NanoDrop 2000c UV-Vis Spectrophotometer.

Ligation of digested DNA.

- **TIMING** 12 hours

1. Prepare a 10 µl ligation reaction using 1 µl of T4 buffer and 1 µl T4 DNA Ligase. Add the DNA of insert and vector in a 1:3 ratio and ligate at 16 °C for 1 hour and then 4 °C overnight.

Transformation of ligated constructs into *E. Coli*

- **TIMING** 19 hours

1. Transform ligation reaction to NEB Turbo Competent *E. coli* cells as previously mentioned.

Confirmation of successful cloning via DNA sequencing

- **TIMING** 38 hours

1. Inoculate 5.5 ml of ampicillin-supplemented LB media with single positive clones using a pipette tip and grow overnight at 37 °C, as previously described.
2. Glycerol stock of cells were created from the grown cultures and stored for future use.
3. The remaining cells were pelleted and purification of plasmid DNA were performed using the commercially available Qiagen Miniprep kit, as mentioned above.
4. The purified DNA was sent to commercial DNA sequencing company to determine the success of cloning (primers used T7 promoter: 5'-TAATACGACTCACTATAGGG-3' and T7 terminator: 5'-GCTAGTTATTGCTCAGCGG-3')

5. The DNA of the clones with confirmation of successful cloning was transformed in BL21Star™ (DE3), which allow for better protein production. Transformation to BL21Star™ (DE3) *E. coli* cells is the same as NEB Turbo Competent *E. coli* cells.

Production of scFv-LPETG constructs

- **TIMING** 25 hours

1. Start a pre-culture by inoculating 10 ml of ampicillin-supplemented LB media with a single clone of the BL21Star™ (DE3) *E. coli* cells, containing the scFv-LPETG. Grow for 8 hours at 37 °C, whilst shaking at 250 rpm.
2. Add 5 ml of the pre-culture in 1 L of ampicillin-supplemented LB media in an Erlenmeyer flask. Grow at room temperature overnight.

Production of sortase A constructs

- **TIMING** 26 hours

1. Start a pre-culture by inoculating 10 ml of ampicillin-supplemented LB media with a single clone of the BL21Star™ (DE3) *E. coli* cells, containing the Sortase A. Grow overnight hours in a shaker at 37 °C, whilst shaking at 250 rpm.
2. Add 5 ml of the pre-culture in 1 L of ampicillin-supplemented LB media in an Erlenmeyer flask. Grow at 37 °C, whilst shaking at 250 rpm, until OD₆₀₀ of 0.8. Induce with 1mM IPTG for 6 hours at 37 °C, whilst shaking at 250 rpm.

Purification of proteins

- **TIMING** 24 hours

1. Centrifuge the cells in 250 ml centrifuge bottles at 6,000g for 15 minutes at 4 °C.
 - **PAUSE POINT** Bacterial pellets may be frozen at -20 °C for several weeks
 2. Discard supernatant and weight the cell pellet. For per gram of pellet, resuspend with 3ml of BugBuster® Master Mix.
 3. Incubate the mixture for 15 minutes at room temperature, whilst rocking gently.
 4. Centrifuge at 15,000g for 30 minutes at 4 °C
 5. Collect supernatant for purification over Ni-NTA Superflow Columns on HPLC.
- * **NOTE** All constructs contain the his₆-tag which enable purification using the Ni-NTA columns.

6. Place fractions of purified proteins into a SnakeSkin Dialysis Tubing and dialyse in PBS without Calcium or magnesium for 4 hours at 4 °C. Change the buffer and dialyse overnight at 4 °C.

■ **PAUSE POINT** The protein may be stored at -80 °C for several months.

▲ **CRITICAL STEP** Avoid freeze/thaw cycles as protein may be negatively affected and lose activity.

Analysis of production and purification

● **TIMING 24 h**

1. Analyse the concentration of the purified protein using Direct Detect™ (Merck Millipore). Add 2 µl of protein on the Direct Detect™ Spotting Tray and use according to manufacturer's protocol. Any other technique to determine the protein concentration can be used as well.

2. Analyse the purified protein using a 12% (v/v) SDS–polyacrylamide gel electrophoresis and Western blotting. Stain the Western blot with anti-6x His tag horseradish peroxidase.

* **NOTE** These experiments evaluate the protein expression level, size and purity of the preparation.

Preparation of scFv-functionalized iron-oxide particles for magnetic resonance imaging (Fig.2I){Ta:2011eq}

● **TIMING 8 hours**

1. Place 2×10^8 particles in a 1.5 ml microcentrifuge tube into the Magnetic Particle Concentrator and wait 2 minutes until all particles are pelleted. Discard the supernatant while the microcentrifuge tube is still in the Magnetic Particle Concentrator.

1. Add 500 µl of water, take the tube out and resuspend the particles.

3. Place the microcentrifuge tube back in the Magnetic Particle Concentrator. Wait for 2 minutes until all particles are pelleted. Discard the supernatant while the microcentrifuge tube is still in the Magnetic Particle Concentrator.

4. Repeat steps 2 and 3 twice with 500 µl DMSO.

5. Resuspend the particle pellet in 120 µl of DMSO.

6. Add 40 µl of 15 mg/ml BOC-HN-GGGWW-COOH peptide to the above particle suspension to a final concentration of 3.8 mmol/l (600 µg peptide).

7. Dissolve 2 mg of PyBOP and 1 mg of DIPEA -in 100 µl DMSO.

▲ **CRITICAL STEP** Prepare the solution fresh.

8. Add 40 µl of the above PyBOP/DIPEA/DMSO mixture to the particle/peptide suspension

in step 6 and incubate the mixture for 2 hours at room temperature whilst shaking.

9. Wash particles three times with deionized water using the Magnetic Particle Concentrator (steps 2 and 3).

10. Resuspend the particles in 60 μ l of 50 % (v/v) TFA in DCM and incubate for 30 min at room temperature whilst shaking to de-protect the amine groups of the conjugated peptide (de-BOC).

! CAUTION TFA and DCM are toxic. Use appropriate gloves and fume hood.

11. Wash particles three times with DCM using magnetic rack (steps 2 and 3).

12. Incubate the particles with 10% (v/v) TEA in DCM at room temperature for 20 min.

13. Wash the particles twice with water and twice with Sortase coupling buffer (steps 2 and 3).

14. Mix scFv-LPETG with Sortase A and GGG-labeled iron-oxide particles in a molar ratio of 1:3:3 in a final volume of 500 μ l sortase reaction buffer and add CaCl_2 to a final concentration of 0.5 mM

15. Incubate the reaction mixture for 3 h at 37°C whilst shaking.

16. Remove excess scFv and Sortase A by washing the particles three times with PBS using the Magnetic Particle Concentrator (steps 2 and 3) and store at 4°C until further use.

■ **PAUSE POINT** These conjugated iron-oxide particles are stable for up to 4 weeks at 4°C.

▲ **CRITICAL STEP** During all incubation steps, place the microcentrifuge tubes in the shaker at a degree of 20-45° to horizontal axis and set shaking speed at 200-230 rpm. Otherwise, particles cannot be suspended well during the reaction.

Expected results: Iron-oxide particles functionalized with anti-LIBS scFv bind to GPIIb/IIIa on immobilized activated platelets whereas iron-oxide particles with control antibody do not bind (Fig.2II).

Preparation of scFv-functionalized layer-by-layer capsules for drug delivery applications (Fig.3I) {Leung:2012hz}

● **TIMING** 2 days

Synthesis of 2-(2-(2-(prop-2-ynyloxy)ethoxy)ethoxy)ethanol

1. A tetrahydrofuran (THF) (50 mL) solution of triethylene glycol (11.6 g, 76 mmol) was prepared in a 100 mL round bottom flask and cooled to 0 °C using an ice/water bath.

2. Sodium hydride (2.0 g, 50 mmol, 60% (w/v) dispersion in mineral oil) was slowly added to the mixture.

3. The mixture was stirred for 20 mins.
4. A dropping funnel was then added to the flask containing propargyl bromide (4.2 ml, 38 mmol).
5. The propargyl bromide was added dropwise to the reaction over 15 mins.
6. The reaction mixture was then stirred at room temperature for 20 h.
7. The final product was concentrated using rotary evaporation.
8. The concentrated product was dissolved in dichloromethane (DCM) (approximately 20 ml) and then washed successively with saturated sodium hydrogen carbonate (NaHCO_3) (2×50 ml) and water (50 ml).
9. The organic phase was dried over magnesium sulfate (MgSO_4) and then the solid residue was removed using filtration.
10. This crude product was then concentrated using rotary evaporation.
11. The crude product was purified by column chromatography, eluting with a 2:3 mixture of *n*-hexane and ethyl acetate (EtOAc) to give 2-(2-(2-(prop-2-ynoxy)ethoxy)ethoxy)ethanol as a light yellow oil (4.5 g, 63%). ^1H NMR (400 MHz, CDCl_3 , tetramethylsilane (TMS)): δH 4.13 (s, 2H, $\text{OCH}_2\text{C}\equiv\text{CH}$), 3.61–3.58 (m, 10H, CH_2O), 3.50 (t, 2H, HOCH_2), 2.75 (br, 1H, HO), 2.38 (s 1H, $\text{CH}\equiv\text{C}$) ppm; ^{13}C NMR (100 MHz, CDCl_3 , TMS) δC 77.2 ($\text{C}\equiv\text{CH}$), 75.6 ($\text{C}\equiv\text{CH}$), 70.4 (CH_2O), 70.3 (CH_2O), 70.1 (CH_2O), 69.6 (CH_2O), 69.2 (CH_2O), 61.5 (HOCH_2), 60.1 ($\text{OCH}_2\text{C}\equiv\text{CH}$) ppm.

Synthesis of 2-(2-(2-(3-(trimethylsilyl)- prop-2-ynoxy)ethoxy)ethoxy)ethanol.

1. Triethylene glycol alkyne (3.39 g, 18 mmol) was added to a 50 mL round bottom flask along with silver chloride (0.24 g, 1.8 mmol) suspended in anhydrous dichloromethane (DCM) (25 ml). The DCM was dried by distillation.
2. 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (3.5 g, 23 mmol) was then added.
3. A sealed 10 ml dropping funnel was attached to the round bottom flask containing chlorotrimethylsilane (2.8 g, 26 mmol).
4. This mixture was stirred at 40 °C in a constant temperature oil bath while chlorotrimethylsilane was added dropwise.
5. The reaction mixture was stirred for 24 h at 40 °C.
6. The mixture was then cooled to room temperature and diluted with 200 ml of *n*-hexane. The organic phase was washed successively with NaHCO_3 (2×50 ml), 0.1 M HCl (2×50 ml) and water (50 ml) using a 100 ml dropping funnel.

7. The organic phase was dried using MgSO_4 and then the solid residue was removed using filtration.

8. The crude product was then concentrated using rotary evaporation.

9. The crude product was finally purified by column chromatography, eluting with a 8:1 mixture of *n*-hexane and EtOAc to give 2-(2-(2-(3-(trimethylsilyl)prop-2-ynoxy)ethoxy)ethoxy)ethanol as a light yellow liquid (2.11 g, 45%). ^1H NMR (400 MHz, CDCl_3 , TMS): δ H 4.13 (s, 2H, $\text{OCH}_2\text{C}\equiv\text{CH}$), 3.61–3.58 (m, 10H, CH_2O), 3.50 (t, 2H, HOCH_2), 2.15 (br, 1H, HO), 0.21 (s, 9H, $\text{Si}(\text{CH}_3)_3$) ppm; ^{13}C NMR (100 MHz, CDCl_3 , TMS) δ C 105.5 ($\text{CH}_2\text{C}\equiv\text{C}$), 91.2 ($\text{C}\equiv\text{CSi}$), 70.8 (CH_2O), 70.7 (CH_2O), 70.3 (CH_2O), 69.1 (CH_2O), 69.0 (CH_2O), 61.1 (HOCH_2), 60.0 ($\text{OCH}_2\text{C}\equiv\text{CH}$), 5.2 (SiCH_3) ppm.

Synthesis of 2-(2-(2-(3-(trimethylsilyl)prop-2-ynoxy)ethoxy)-ethoxy)ethyl methacrylate (TMSPgTEGMA)

1. TMS-alkyne triethylene glycol (2.0 g, 7.7 mmol) and triethylamine (0.81 g, 8 mmol) were measured out in small glass vials and added to a two necked round bottom flask using excess DCM (50 ml).

2. Methacryloyl chloride (0.78 ml, 8 mmol) dissolved in DCM (10 ml) was added into a 50 ml equilibrating dropping funnel. The funnel was placed in one neck with a septum at the top containing an outlet needle. The second neck also contained a septum with a needle which slowly bubbled nitrogen gas into the solution.

3. The experimental setup was cooled to 0 °C using an ice/water bath.

▲ CRITICAL STEP The methacryloyl chloride solution was then added very slowly over 1 hr with constant stirring. Care must be taken to add this slowly as excess reactants can cause polymerization to occur.

5. The reaction mixture was then stirred at room temperature for 18 h and then filtered to remove triethylamine hydrochloride.

6. The filtrate was washed with saturated NaHCO_3 (2 × 50 ml) and water (50 ml) using a 200 ml separating funnel.

7. The organic phase was dried using MgSO_4 and then filtered to form a yellowish brown solution.

8. This solution was concentrated using rotary evaporation.

9. The crude brown oil was purified by column chromatography, eluting DCM to give 2-(2-(2-(3-(trimethylsilyl)prop-2-ynoxy)ethoxy)-ethoxy)ethyl methacrylate (TMSPgTEGMA) as a light yellow liquid (2.1 g, 88%). ^1H NMR (400 MHz, CDCl_3 , TMS): δ H 6.15 (s 1H, CH_2

= C(CH₃)), 5.51 (s 2H, CH₂ = C(CH₃)), 4.13 (s, 2H, OCH₂C≡CH), 3.61–3.58 (m, 10H, CH₂O), 3.50 (t, 2H, HOCH₂), 2.0 (s, 3H, CH₂ = C(CH₃)), 0.21 (s, 9H, Si(CH₃)₃) ppm; ¹³C NMR (100 MHz, CDCl₃, TMS) δC 168.3 (CO), 133.8 (= CCH₃), 131.5 (= CH₂), 106.1 (CH₂C≡C), 91.6 (C≡CSi), 70.3 (CH₂O), 70.2 (CH₂O), 70.0 (CH₂O), 69.4 (CH₂O), 69.2 (CH₂O), 61.8 (HOCH₂), 60.2 (OCH₂C≡CH), 18.8 (= CCH₃), 4.1 (SiCH₃) ppm.

Synthesis of Alkyne-Functionalized Copolymers Poly(MEO₂MA- co-OEGMA-co-PgTEGMA)

1. Copper(I) chloride (6.5 mg, 0.071 mmol) and 2,2'-bipyridine (20.3 mg, 0.14 mmol) were measured out precisely and added to a 10 ml round bottom flask.
2. Methyl 2-bromo propionate (10.1 mg, 0.071 mmol), OEGMA (271 mg, 0.57 mmol), MEO₂MA (1089 mg, 6.2 mmol) and TMSPgTEGMA (108 mg, 0.36 mmol) were added to small glass vials and then transferred to the 10mL round bottom flask using 1.4 ml of ethanol.
3. The mixture was dissolved by gentle stirring, which was maintained throughout the reaction.

▲ **CRITICAL STEP** The round bottom was then sealed with a septum with a long needle inserted into the solution and smaller needle providing an outlet for the gas. The solution was gently bubbled with argon for half an hour. Bubbling should not be too aggressive or partial evaporation of the solution will occur.

5. The needles were then removed and the mixture was heated to 60 °C with constant stirring, this was done using a constant temperature oil bath.
6. The solution was polymerized for 16 h.
7. MilliQ water (20 ml) was then added to the mixture and then it was placed in a dialysis tube.
8. This tube was then placed in a 200 ml beaker containing milliQ water. The water was changed 9 times leaving the product as a pale green solution.
9. This solution was finally dried using rotary evaporation under increased pressure (5 mbar), temperature was slowly increased until the solution began bubbling but care must be taken to avoid aggressive bubbling or bumping.
10. The polymer was characterized by NMR and gel permeation chromatography (GPC). The GPC analysis (THF eluent) showed the polymer had $M_n = 25$ kDa, $M_w/M_n = 1.5$. The slightly higher polydispersity was thought to be due to the loss of the protecting group during polymerization. The alkyne percentage was approximately 5%, as determined by NMR (ratio of peak at 4.1 ppm (2H) to 2.5 ppm (1H).)

Synthese of scFv-functionalized layer-by-layer capsules

● TIMING 2 days

1. Place 50 μl of a 5 wt % suspension of 3 μm -diameter SiO_2 particles in a 1.7 ml eppendorf centrifuge tube and centrifuge at 1000 x g for 60 s. Remove the supernatant and replace with 250 μl of 50 mM pH 4.0 acetate buffer. Ensure the pellet is well agitated before repeating the centrifugation/redispersion wash cycle twice. Resuspend the particles in 50 μl of 50 mM pH 4.0 acetate buffer.

▲ **CRITICAL STEP** The particles must be well suspended before each polymer layer is added. Failure to do this will result in aggregated particles.

2. Add 200 μl of PVPON solution to the resuspended particles. Allow the polymer to adsorb to the surface of the particles for 15 min with constant agitation. Centrifuge the particles at 1000 x g for 60 s and remove the supernatant and replace with 250 μl of 50 mM pH 4.0 acetate buffer. Ensure the pellet is well agitated and repeat the centrifugation/redispersion wash cycle twice before resuspending the particles in 50 μl of 50 mM pH 4.0 acetate buffer.

3. Add 200 μl of PMA solution to the resuspended particles. Allow the polymer to adsorb to the surface of the particles for 15 min with constant agitation. Centrifuge the particles at 1000 x g for 60 s and remove the supernatant and replace with 250 μl of 50 mM pH 4.0 acetate buffer. Ensure the pellet is well agitated before repeating the centrifugation/redispersion wash cycle twice. Resuspend the particles in 50 μl of 50 mM pH 4.0 acetate buffer.

4. Add 200 μl of PVPON_{Alk} solution to the resuspended particles. Allow the polymer to adsorb to the surface of the particles for 15 min with constant agitation. Centrifuge the particles at 1000 x g for 60 s and remove the supernatant and replace with 250 μl of 50 mM pH 4.0 acetate buffer. Repeat the centrifugation/redispersion wash cycle twice before resuspending the particles in 50 μl of 50 mM pH 4.0 acetate buffer.

5. Repeat steps 3 and 4 until 6 layers of PMA and 5 layers of PVPON_{Alk} have been deposited (so PMA is the last layer deposited). Resuspend the particles in 50 μl of 150 mM pH 5.0 acetate buffer.

6. Add 200 μl of PEG_{Alk} solution to the resuspended particles. Allow the polymer to adsorb to the surface of the particles for 15 min with constant agitation. Centrifuge the particles at 1000 x g for 60 s and remove the supernatant and replace with 250 μl of 150 mM pH 5.0 acetate buffer. Repeat the centrifugation/redispersion wash cycle twice before resuspending the particles in 50 μl of 150 mM pH 5.0 acetate buffer.

7. Add a mixture of 150 μ l of the bisazide crosslinker, 50 μ l of sodium ascorbate and 50 μ l of copper sulphate to the resuspended particles. Incubate the particles overnight with constant agitation.

8. Centrifuge the particles at 1000 x g for 60 s and remove the supernatant and replace with 250 μ l of 150 mM pH 5.0.

▲ **CRITICAL STEP** When centrifuging both capsules and particles, the ‘soft’ mode of the centrifuge must be enabled. This slows the acceleration and deceleration during the centrifugation step and avoids/minimises aggregation.

9. Ensure the pellet is well agitated before repeating the centrifugation/redispersion wash cycle twice. Resuspend the particles in 50 μ l of 150 mM pH 5.0 acetate buffer.

10. Add a mixture of 150 μ l of the Sortase peptide (GGG-PEG-Az), 50 μ l of sodium ascorbate and 50 μ l of copper sulfate to the resuspended particles. Incubate the particles for 30 min with constant agitation.

▲ **CRITICAL STEP** Prepare fresh solutions of sodium ascorbate and copper sulphate as they can show reduced activity when stored overnight.

11. Centrifuge the particles at 1000 x g for 60 s and remove the supernatant and replace with 250 μ l of 150 mM pH 5.0 acetate buffer. Resuspend the particles in 200 μ l of 150 mM pH 5.0 acetate buffer. Repeat the centrifugation/redispersion wash cycle twice before resuspending the particles in 50 μ l of 50 mM pH 4.0 acetate buffer.

▲ **CRITICAL STEP** Peptide functionalized particles have a tendency to aggregate during dissolution of the silica core, therefore protective capping layers are required to maintain colloidal stability.

12. Add 200 μ l of PMA solution to the resuspended particles. Allow the polymer to adsorb to the surface of the particles for 15 min with constant agitation. Centrifuge the particles at 1000 x g for 60 s and remove the supernatant and replace with 250 μ l of 50 mM pH 4.0 acetate buffer. Repeat the centrifugation/redispersion wash cycle twice before resuspending the 13. particles in 50 μ l of 50 mM pH 4.0 acetate buffer.

13. Add 200 μ l of PVPON solution to the resuspended particles. Allow the polymer to adsorb to the surface of the particles for 15 min with constant agitation. Centrifuge the particles at 1000 x g for 60 s and remove the supernatant and replace with 250 μ l of 50 mM pH 4.0 acetate buffer. Repeat the centrifugation/redispersion wash cycle twice before resuspending the particles in 50 μ l of 50 mM pH 4.0 acetate buffer.

14. Repeat step 12. Resuspend the particles in 200 μ l of 50 mM pH 4.0 acetate buffer.

!CAUTION Hydrofluoric acid and ammonium fluoride are very toxic. Extreme care should be taken when handling HF solution, and only small quantities should be prepared.

15. To dissolve the sacrificial silica core, add 150 μl of pH 5.0 buffered HF to the 100 μl of well suspended particles. Allow the silica to dissolve for 60 s with constant gentle agitation. The solution should become clear.


16. Centrifuge the particles at 1500 x g for 4 min and remove the supernatant and replace with 250 μl of 50 mM pH 4.0 acetate buffer. Repeat the centrifugation/redispersion wash cycle twice. Resuspend the capsules in 200 μl of PBS. Store the particles in PBS, with constant agitation, for 12 hours to remove the PMA / PVPO protecting layers to form peptide functionalized LbL capsules.

17. To immobilize scFv-LPETG, incubate the GGG-PEG-Az-functionalized capsules in 150 μl of Sortase reaction buffer (pH 8.0) with Sortase A, scFv-LPETG and CaCl_2 . Add Sortase A, scFv-LPETG and CaCl_2 to a final concentration of 0.1 g/l, 0.1 g/l and 0.5 mM, respectively. Incubate the mixture at 37 °C for 1 h with gentle shaking. After conjugation, wash the scFv-LPETG-functionalized capsules with PBS to remove any excess antibody and Sortase.

▲ **CRITICAL STEP** During all polymer adsorption steps the particles should be constantly agitated using either a thermomixer or rotary mixer.

Expected results: LbL capsules functionalized with scFv bind to GPIIb/IIIa on activated platelets in a thrombus whereas LbL capsules with control antibody do not bind (Fig.3II). If capsules of a different size are required, then the amount of polymer, cross-linker, peptide and other reagents should be scaled in proportion to surface area. For example, if you take the same mass of 1 μm and 3 μm diameter particles, the total surface area of the 1 μm sample will have 3 times the surface area of 3 μm sample. Therefore the 1 μm sample will require 3 times the amount of polymer, cross linker and peptide.

? TROUBLESHOOTING

Problem	Solution
DNA is not digested fully	<ul style="list-style-type: none"> • Check that the restriction enzymes are working by performing single digest.
No colonies on the LB-agar plates after transformation	<ul style="list-style-type: none"> • Check that cells were thawed on wet ice and not standing for too long. • Check with commercially available positive DNA such as PUC19 to determine the competency of cells.
Re-ligated vector colonies on the LB-agar plates after transformation	<ul style="list-style-type: none"> • Double check that the restriction enzymes are working and that there is good separation on the agarose gel before using the scalpel blade to cut out the DNA band of interest.
No scFv conjugation	<ul style="list-style-type: none"> • Check if GGG has been properly installed. • Check Sortase activity. • Check accessibility of LPETG tag on antibody. • Check the correct Ca^{2+} concentration has been used (0.5mM). ScFv precipitate out of solution at the higher Ca^{2+} concentrations (see picture).
	
LbL particles have aggregated	<ul style="list-style-type: none"> • Ensure the particles are well dispersed before adding the polymer or antibody solution • Ensure the particles are agitated during polymer and antibody deposition • Ensure the centrifugation speeds used do not irreversibly pellet the particles

No capsules were formed	<ul style="list-style-type: none">• Ensure each polymer layer was deposited under the conditions described• Ensure the cross-linking reaction was performed with fresh solutions of sodium ascorbate and copper sulfate• Capsules require higher centrifugation speeds for a longer time. Make sure the centrifugation speeds are observed.
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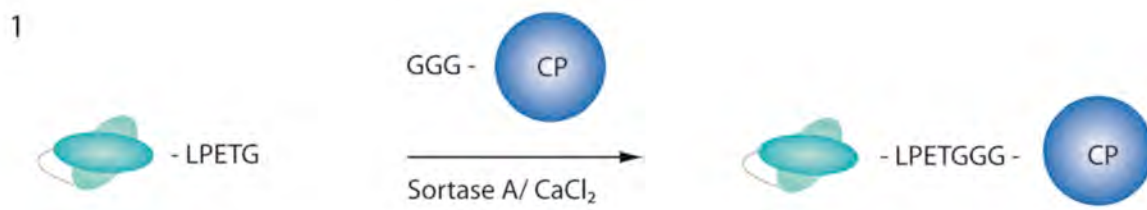


Figure 1: Schematic illustration of the conjugation process between scFv-LPETG and the GGG-coupling partner (CP)

Site-specific labeling of scFv-LPETG by Sortase mediated transpeptidase reaction. Sortase A recognizes substrate with an LPXTG motif, cleaving the peptide bond between the threonine and glycine and resulting in a thioacyl intermediate. The modified (oligo)glycine partner (CP) links to the targeting scFv via peptide linkage.

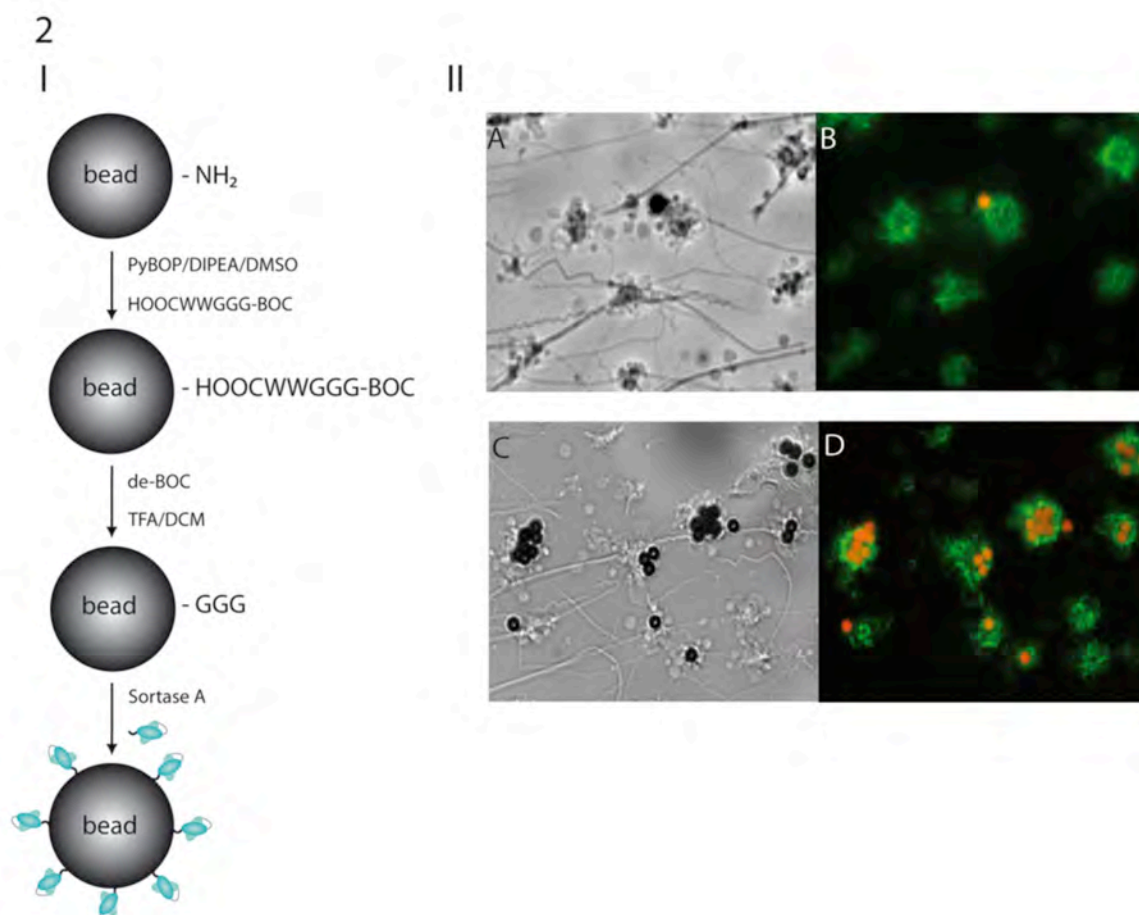


Figure 2: Generation and specific binding evaluation of scFv-coupled iron-oxide particles to platelet aggregates in a flow chamber system

I) Magnetic particles of iron oxide (MPIO beads) with amine surface groups are modified with tert-butyloxycarbonyl-protected GGGWW peptides via carboxyl activation and deprotected. Subsequently the particles are site-specifically labeled with scFv-LPETG by Sortase mediated transpeptidase reaction. II) (A) Particle binding under shear conditions is evaluated in a flow chamber system. Differential interference contrast (DIC) and (B) fluorescence image show the lack of binding of the control particles (DIC image: black; fluorescence image: orange or yellow auto-fluorescence). Platelet aggregates (DIC: white or gray; fluorescence: green indicates platelet membrane) formed by aggregation of activated platelets on collagen-1 fibers were coated on the flow chamber surface. Platelet membranes were specifically stained with PAC-1-FITC directed toward GPIIb/IIIa receptors and appears as green fluorescence. (C) DIC and (D) fluorescence image show the strong binding of the scFv-coupled particles.

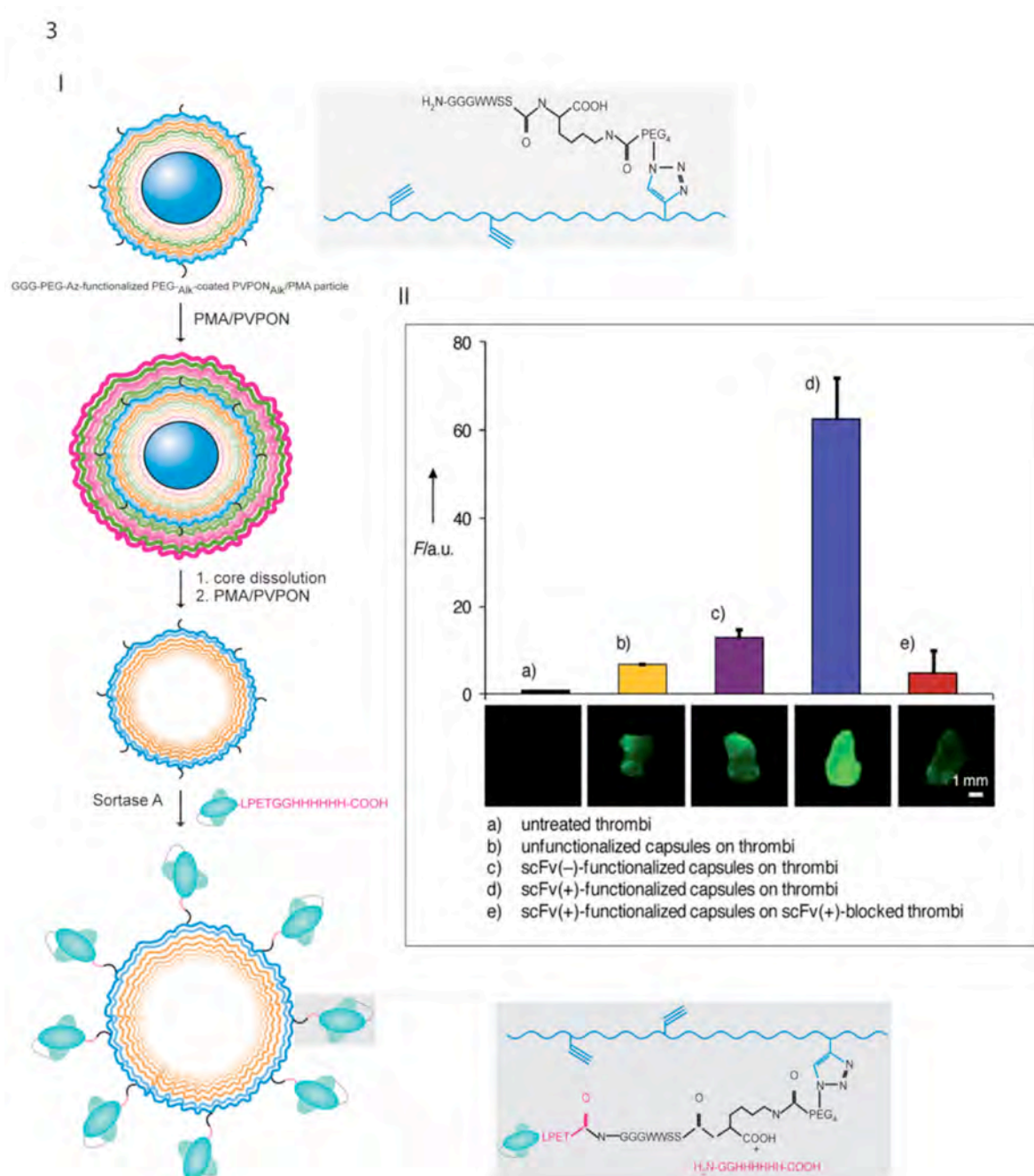


Figure 3: Generation and targeting of sortase mediated scFv-functionalized LbL capsules to platelet rich thrombi

I) Schematic illustration of scFv-LbL capsules generation. II) Targeting experiments to platelet rich thrombi. **a)** non-treated thrombi **b)** non-functionalized (PVPN_{Alk})₅/PEG_{Alk} capsules incubated with thrombi **c)** capsules conjugated to mutated control scFv **d)** capsules functionalized with platelet binding scFv and **e)** preincubation of thrombi with blocking non-

labelled scFv reduces binding to control levels. Targeting experiments were conducted for 30 min at 37 °C in pH 7.2 PBS (with $\text{Ca}^{2+}/\text{Mg}^{2+}$) and analyzed by near infrared imaging. The fluorescence intensity of untreated thrombi was set at 1.

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Author contributions

C.E.H. designed and produced the antibodies, supervised the work and oversaw preparation of the manuscript, K.A. prepared the manuscript and made the figures, A.P.R.J. generated the nanocapsules and supervised the LbL capsule work, G.K.S. made the polymers for the nanocapsules, H.T.T. performed the MPIO, M.K.M.L. performed the nanocapsule and thrombus targeting work, S.P. established Sortase mediated antibody conjugation, X.W. made scFv antibody, F.C. supervised the nanocapsule work, K.P. supervised the MPIO work. All authors contributed to writing of the manuscript and approved the final version.

References