In situ regeneration of bioactive coatings enabled by an evolved *Staphylococcus aureus* sortase A

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Surface immobilization of bioactive molecules is a central paradigm in the design of implantable devices and biosensors with improved clinical performance capabilities. However, *in vivo* degradation or denaturation of surface constituents often limits the long-term performance of bioactive films. Here we demonstrate the capacity to repeatedly regenerate a covalently immobilized monomolecular thin film of bioactive molecules through a two-step stripping and recharging cycle. Reversible transpeptidation by a laboratory evolved *Staphylococcus aureus* sortase A (eSrtA) enabled the rapid immobilization of an anti-thrombogenic film in the presence of whole blood and permitted multiple cycles of film regeneration *in vitro* that preserved its biological activity. Moreover, eSrtA transpeptidation facilitated surface re-engineering of medical devices *in situ* after *in vivo* implantation through removal and restoration film constituents. These studies establish a rapid, orthogonal and reversible biochemical scheme to regenerate selective molecular constituents with the potential to extend the lifetime of bioactive films.

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Medical devices in blood-contacting applications, such as stents, heart valves, ventricular assist devices and extracorporeal support systems, as well as vascular grafts and access catheters are prone to failure due to maladaptive host responses at the blood–material interface. Immobilization of bioactive molecules and drug eluting thin films on implantable devices have yielded promising combination products that abrogate thrombotic cascades and detrimental inflammatory reactions, enhance device integration and local tissue repair, and inhibit microbial colonization. However, the clinical translation of these strategies remains constrained by the limited therapeutic duration afforded by a finite reservoir of bioactive agents, as well as by the degradation or denaturation of surface components by oxidation, hydrolysis and proteolysis when exposed over time to a physiological environment. Efforts to improve the stability and activity of thin film constituents for both biomedical and biotechnological applications have included attempts to judiciously alter surface properties, such as hydrophilicity, charge, topography and immobilization chemistry, as well as through rational and evolutionary protein engineering. Despite recent progress in these areas, a surface coating for implantable devices that reliably retains biological activity over extended clinically relevant timescales has not been developed.

In principle, enzyme ligation offers an opportunity for catalysing reversible bond-forming reactions that could enable the molecular regeneration of bioactive thin films in situ. Staphylococcus aureus sortase A (SrtA) catalyses the covalent transpeptidation of a C-terminal ‘sorting motif’ LPXTG to N-terminal oligoglycine (for example, GGG) nucleophiles through an acyl-enzyme complex forming an LPXTG-GGG bond. Because of its synthetic simplicity and the very limited occurrence of the LPXTG motif in native proteins, SrtA-catalysed transpeptidation has been broadly applied in protein purification, labelling and immobilization onto solid supports. However, the low catalytic activity and substrate affinity of wild-type (WT) SrtA necessitates high molar excesses of the enzyme and long incubation times to approach reaction completion, limiting its effectiveness and applicability. An evolved SrtA mutant (eSrtA) has recently been generated that exhibits 120-fold higher LPETG-ligation activity than the WT enzyme. This enzyme suggested the possibility of multiple rapidly catalysed cycles of removal and reversible assembly of bioactive molecular films onto oligoglycine-modified surfaces both in vitro and in vivo. Indeed, we observed that eSrtA transpeptidation was reversible, enabling multiple cycles of peptide bond formation and cleavage, and, thereby, facilitating film regeneration in the presence of whole blood in vitro and in vivo. These studies establish a rapid, orthogonal and reversible scheme, which can be used to regenerate selective molecular constituents with the potential to extend the lifetime of bioactive films, or likewise, as a method to load and release any of a number of material bound constituents for controlled drug loading and delivery, or as a strategy for material dissolution.

Results
An evolved sortase catalyses rechargeable molecular assembly.
We have extensively characterized the ability of thrombomodulin (TM) to limit tissue factor induced thrombin production under venous and arterial flow conditions using both experimental and computational models. Surfaces functionalized with TM, a major physiological inhibitor of thrombin generation, exhibited reduced thrombogenicity in vitro and in vivo, thereby, supporting its potential utility in blood-contacting devices. To demonstrate the ability to reversibly assemble TM on oligoglycine surfaces in a site-specific manner (Fig. 1a), we first generated a recombinant human TM fragment containing a C-terminal LPETG motif (TMLPETG). WT sortase (WT SrtA) and an eSrtA produced by directed evolution using a yeast display system were generated, as previously reported. Model pentaglycine surfaces, which match the natural SrtA substrate on the S. aureus cell wall, were constructed by immobilizing a biotinylated pentaglycine peptide (NH2-GGGGK-GGK-biotin) on surfaces pre-coated with streptavidin. Surface immobilization of TMLPETG using eSrtA yielded a ~10-fold higher surface density than that attained in the presence of an equivalent concentration of WT SrtA. Further, despite a 20-fold increase in WT SrtA, only a modest increase in TM surface density was observed (Fig. 1b).

Near-complete removal of immobilized TM films could be achieved with eSrtA in 30 min, whereas WT SrtA was substantially less efficient under identical conditions (Fig. 1c). In this system, the binding kinetics of TMLPETG by eSrtA roughly approximated those of biotin-streptavidin (Fig. 1d). As illustrated by 10 repetitive stripping and charging cycles using eSrtA, rapid and reproducible regeneration of surface-bound TM films proved feasible with preservation of surface catalytic activity and sustained production of activated protein C (aPC) (Fig. 1e,f).

Repetitive molecular recharging can be performed in blood.
Because of the rarity of LPETG and GGG motifs in native proteins, we postulated that the two-step strip/recharge reaction mediated by eSrtA would provide a feasible in vivo strategy to selectively regenerate molecular constituents on or within medical implants in situ. As proof-of-concept, we demonstrated the ability to immobilize TMLPETG on pentaglycine surfaces in 50% v/v whole blood at 37°C. We observed that eSrtA was capable of efficiently functionalizing model surfaces greater than 20-fold more effectively than WT SrtA (Fig. 3a). In addition, eSrtA was capable of functionalizing surfaces in whole blood at levels equivalent to that achieved in defined reaction buffer with only a five fold increased concentration (0.1 equivalents versus 0.5 equivalents). We performed repeated cycles of striping/recharging in 50% v/v whole blood and observed both efficient removal and regeneration of TM. Significantly, aPC generation after five rounds of charging was equivalent to that observed on our initial model surface (Fig. 3b,c). These results underscore the enhanced catalytic activity of eSrtA as a critical enabling step in the application of a two-step strip/recharge scheme for rapid and repetitive regeneration of biologically active molecular films.

Sortase mediates molecular assembly and disassembly in vivo.
Polyurethane venous access catheters are widely used, but continue to be limited by a risk of thrombosis and infection, despite attempts to limit these risks by surface immobilization of bioactive molecular films. Venous catheters are available in sizes compatible for small animal studies, they provide a convenient model system to assess stripping and recharging of surface-bound biologically active molecules in vivo. Initial investigations focused on the application of eSrtA transpeptidation to immobilize and remove LPETG-tagged probes on pentaglycine-modified polyurethane catheters deployed in the mouse inferior vena cava. Catheters were modified with...
cyclooctyne motifs using a sequential scheme (Supplementary Fig. 1), as previously reported31,37, and azide reactivity was confirmed using rhodamine azide probes (Supplementary Fig. 2). An azide-tagged pentaglycine peptide (NH₂-GGGGG-N₃) was used to generate pentaglycine motifs on catheters. Reaction parameters for eSrtA-mediated attachment and removal of a biotin-LPETG probe on catheters were first determined under in vitro conditions (Supplementary Figs 3–5). Using 6–8-week-old C57BL/6 mice, pentaglycine-modified catheters were inserted via the femoral vein into the inferior vena cava, followed by intravenous administration of eSrtA with either biotin-LPETG or triglycine (GGG) to charge or strip the catheter surface, respectively (Fig. 4a). Analysis of explanted catheters confirmed the selective immobilization or removal of biotin-LPETG probes within 1 h (Fig. 4b,c and Supplementary Figs 6–9). In a parallel set of experiments using catheters bearing Alexa Fluor 750-LPETG, real-time fluorescent imaging confirmed that the surface-bound near-IR probe was nearly completely removed within 1 h after intravenous administration of eSrtA and GGG (Fig. 4d,e).

To determine whether nonspecific adsorption of plasma proteins or other blood components limits surface accessibility to systemically delivered eSrtA, catheters were deployed in the jugular vein for 7 days using a rat model. Pentaglycine-derivatized polyurethane catheters were inserted and after 1 week, Texas Red-labelled TM₈PET₈G was administered intravenously with or without eSrtA. Catheters were explanted 1 h later and TM surface conjugation was confirmed using fluorescence microscopy (Fig. 5a and Supplementary Fig. 10). In situ stripping of Texas Red-TM₈PET₈G and recharging efficiency was examined in a similar manner. Specifically, pentaglycine catheters functionalized with Texas Red-TM₈PET₈G were deployed within the jugular vein for 7 days. Catheters were removed 1 h after intravenous administration of GGG with or without eSrtA and stripping verified by fluorescence imaging (Fig. 5b and Supplementary Fig. 10). In situ recharging was assessed by first initiating the in vivo removal of TM₈PET₈G from catheter surfaces after deployment within the rat jugular vein for 7 days, as detailed above, followed 24 h later by intravenous administration of Texas Red-TM₈PET₈G with or without eSrtA. Significantly, TM surfaces could be charged, stripped and once again recharged with TM to levels observed before catheter implantation (Fig. 5b,c and Supplementary Fig. 10).

**Figure 1 | Sortase-catalysed rechargeable surface assembly.** (a) Two-step ‘rechargeable’ surface assembly cycle initiated by sortase-catalysed charging of LPETG-tagged biomolecules on pentaglycine-modified surfaces, followed by sortase-catalysed stripping to regenerate pentaglycine anchor sites for additional surface reaction cycles. (b) Immobilization of 1 μM TM₈PET₈G on pentaglycine-coated microwells using 0.1 molar equivalents evolved sortase (eSrtA), 0.1 and 2 molar equivalents WT SrtA, or no sortase as a negative control. (c) Following immobilization of 1 μM TM₈PET₈G on pentaglycine-coated microwells using 0.1 molar equivalents eSrtA, removal of bound TM was carried out using 20 μM of either evolved eSrtA or WT SrtA with 1 mM triglycine. (d) Sortase-catalysed binding of TM₈PET₈G on pentaglycine-modified model surfaces following 1 h and 16 h reactions were compared with the binding of biotinylated TM (TM-biotin) directly on streptavidin-coated microwells. In parallel, TM₈PET₈G was incubated in microwells without sortase as a negative control. (e) Rapid repetitive recharging of TM₈PET₈G on pentaglycine surfaces by eSrtA. Sequential eSrtA-catalysed charging (red) and stripping (blue) cycles of TM₈PET₈G on pentaglycine surfaces (n = 4). (f) Co-factor activity of immobilized TM to catalyse production of activated protein C (aPC) following the initial assembly of TM₈PET₈G, stripping by eSrtA, and regeneration of TM₈PET₈G by eSrtA. **P < 0.01, ***P < 0.001 (Student’s t-test), error bars denote s.d. (n ≥ 3).
Discussion

Current techniques to covalently modify surfaces with bioactive compounds have largely involved bioconjugation schemes that link nucleophilic motifs such as amines, thiols and hydroxyls to respective partner electrophiles. The abundant presentation of these motifs in the complex chemical landscape of biological systems reduces the efficiency of targeting payloads to regenerate device reservoirs or surfaces. Recent efforts to modify blood-contacting surfaces with bioactive molecules have led to the application of a variety of bio-orthogonal coupling chemistries, notably Staudinger ligation and native chemical ligation, as well as click cycloaddition and intein-mediated splicing. Nonetheless, even under controlled material surfaces that can be selectively and rapidly ‘recharged’ enabling technology for this concept. We confirmed the capacity of eSrtA to catalyse multiple cycles of rapid assembly and removal of LPETG-tagged biomolecules and established that such a
strategy could be performed in the presence of whole blood in vitro and in vivo. Taken together, our findings establish the capacity to rapidly and reproducibly regenerate selective molecular constituents after device implantation with the potential to substantially extend the lifetime of bioactive films and enhance clinically related performance characteristics. It is anticipated that such a strategy could be applied to stripping and regenerating any of a number of potential material bound constituents that display finite stability or activity.

Methods

Materials. Unless otherwise specified, all reagents were purchased from Sigma-Aldrich and used without further purification. The peptides NH₂-GGGGG-NH₂, NH₂-GGGGGK-biotin, biotin-LPETG and NH₂-LPETG were obtained from GenScript.

Synthesis of GGG-PEG5k-biotin. A total of 100 mg of biotin-PEG(5k)-NH₂ (Creative PEG works) and 32.3 mg Fmoc-Gly-Gly-Gly-COOH (Santa Cruz Biotechnology) were dissolved in 500 ml anhydrous dichloromethane. A total of 22.3 mg of HBTU and 12.0 mg of hydroxybenzotriazole were dissolved in 1 ml of N,N-diisopropylethylamine and then reacted to oxidative inactivation.

Leu, which has been previously shown to result in a construct that is more resistant to enzymatic cleavage. Met-388 was mutated to expression and purification of TMLPETG and sortase variants are provided in the Supplementary Methods section (Supplementary Table 1). Met-388 was mutated to Leu, which has been previously shown to result in a construct that is more resistant to oxidative inactivation.

Expression of TMLPETG and sortase variants. Detailed DNA sequence, bacterial expression and purification of TMLPETG and sortase variants are provided in the Supplementary Methods section (Supplementary Table 1). Met-388 was mutated to Leu, which has been previously shown to result in a construct that is more resistant to oxidative inactivation.

Preparation of fluorescently labelled TMLPETG. TM was modified with an amine-reactive fluorescent probe. A total of 4.0 ml of TMLPETG stock in PBS (1 mg ml⁻¹) was prepared at 4°C and 100 ml of Texas Red NHS ester (Invitrogen) at 10 mM DMSO was added dropwise. The mixture was reacted for 1 h at room temperature with stirring followed by an overnight incubation at 4°C. The solution was then dialyzed against PBS at 4°C for 72 h to remove unreacted fluorescent dye and passed through a PD10 column with TBS buffer (25 mM Tris, 150 mM NaCl, pH 7.5). The purified Texas Red-TM conjugate was analyzed by UV–vis spectroscopy and SDS–polyacrylamide gel electrophoresis performed by illumination on a standard UV light box.
**Figure 5** *In situ* molecular recharging of venous catheters after *in vivo* deployment. (a) To characterize *in situ* charging of a chronically implanted venous catheter, pentaglycine-modified polyurethane catheters were initially deployed in the rat jugular vein for 7 days. Texas Red-labelled TMPEG was or without eSrtA was systemically administered by intravenous injection via the dorsal penile vein. Catheters were removed 1 h later and imaged using fluorescence microscopy. Representative images are shown (scale bar, 1 mm). (b) To characterize *in situ* stripping, pentaglycine catheters linked to Texas Red-TM were deployed in the rat jugular vein for 7 days. GGG with or without eSrtA was delivered intravenously and 1 h later catheters were removed and imaged. To examine *in situ* recharging, Texas Red-TM catheters were first stripped by intravenous administration of GGG and eSrtA and 24 h later, Texas Red-TMPEG and eSrtA was administered intravenously. Catheters were removed 1 h later for imaging. Representative images are shown (scale bar, 1 mm). (c) Catheter fluorescence intensity was quantified using ImageJ and plotted as. *P*<0.05, **P*<0.01, ***P*<0.001 (Student’s *t*-test), error bars denote s.d. (*n*≥3). A.U., arbitrary units.

**Immobilization of TMPEG on model substrates.** Direct sortase-mediated immobilization of TMPEG on pentaglycine surfaces was tested in streptavidin-coated 96-well microplates (Pierce). A total of 200 μM NH₂-GGGGG-biotin or biotin-PEG5k-GGG was incubated for 1 h in each well, and washed with TBS (20 mM Tris, 100 mM NaCl, pH 7.5) with 0.05% v/v Tween 20. Various concentrations of TMPEG were immobilized using 0.1–2 molar equivalents of WT SrtA or eSrtA for 1 h at room temperature. All sortase reactions were performed in a defined reaction buffer consisting of 25 mM Tris-HCl, 150 mM NaCl, 0.5 mM CaCl₂, pH 7.5. Stripping of immobilized TM was performed using 20 μM SrtA and 1-mM GGG peptide. SrtA-catalysed assembly of TMPEG was performed in heparinized (HDI) and 4% v/v triethylamine (TEA) in toluene for 1 h at 50°C. Isocyanate activated catheters were then reacted overnight at 25°C with 1 mg ml⁻¹ dibenzocyclooctyne (DBCO)-amine linker (Click Chemistry Tools) and TEA (1% v/v) in toluene, rinsed with toluene for 6 h, and dried overnight under vacuum at 25°C. For rat studies, catheter lumens were heat sealed before surface modification and after isocyanate activation, as described above, catheters were reacted overnight with 2 mg ml⁻¹ DBCO-PEG2k-amine linker (Nanocs) and TEA 1% v/v in acetonitrile at 40°C and then rinsed with acetonitrile for 3 h. To confirm surface cyclooctyne reactivity, DBCO-activated catheters were reacted with 1 mg ml⁻¹ tetramethyl-rhodamine-5-carboxyl azide (Invitrogen) in 1:4 tert-butanol/PBS at 37°C for 24 h followed by rinsing in methanol for 24 h.

The quantity of TM immobilized in each well was determined by an ELISA assay per manufacturer’s instructions (American Diagnostica). Surface activity of immobilized TM was determined by incubating 100 μl of 0.2 μM human protein C (Callbiochem, Gibbstown), 5 mM calcium chloride and 2 mM human α-thrombin (Haematologic Technologies) in Tris buffer (20 mM Tris, 100 mM NaCl, pH 7.5) with 0.1% bovine serum albumin at 37°C for 1 h. Activated protein C generation was determined using an enzymatically digestible chromogenic substrate Spectrozyme PCa (Sekiisi Diagnostics). Experiments from three independent replicates were averaged.

**Modification of catheters *in vitro*.** Polyurethane catheters (1 Fr, 2 cm length, SAI Infusion) were derivatized with pentaglycine motifs. For mouse studies, inner and outer catheter surfaces were reacted with 16% v/v hexamethylene disocyanate (HDI) and 4% v/v triethylamine (TEA) in toluene for 1 h at 50°C and then rinsed in toluene for 6 h. Isocyanate activated catheters were then reacted overnight at 40°C with 1 mg ml⁻¹ dibenzocyclooctyne (DBCO)-amine linker (Click Chemistry Tools) and TEA (1% v/v) in toluene, rinsed with toluene for 6 h, and dried overnight under vacuum at 25°C. For rat studies, catheter lumens were heat sealed before surface modification and after isocyanate activation, as described above, catheters were reacted overnight with 2 mg ml⁻¹ DBCO-PEG2k-amine linker (Nanocs) and TEA 1% v/v in acetonitrile at 40°C and then rinsed with acetonitrile for 3 h. To confirm surface cyclooctyne reactivity, DBCO-activated catheters were reacted with 1 mg ml⁻¹ tetramethyl-rhodamine-5-carboxyl azide (Invitrogen) in 1:4 tert-butanol/PBS at 37°C for 24 h followed by rinsing in methanol for 24 h.
DBCO activated catheters were reacted overnight with NH$_2$-GGGGG-N$_2$ at 37 °C and rinsed with TBS buffer. Pentaglycine-modified catheters were incubated with biotin-LPETG or LPETG-labelled Alexa Fluor 750 peptide (2 to 100 μM) and 0.1 molar equivalents of eSrtA relative to the LPETG probe concentration for 30 min or 1 h and rinsed for 4 h in TBS. Biotinylated catheters were incubated in 0.1 mg ml$^{-1}$ Cy3-labelled streptavidin in TBS with 0.05% Tween 20 for 30 min and rinsed overnight in TBS. Imaging of catheters was carried out using the Zeiss Discovery V20 Stereo Microscope and quantitative image analysis by ImageJ to determine fluorescence intensity.

**In situ modification of catheters deployed in mice.** All animals were conducted under the approval of the BDIDMC Animal Care and Use Committee. Pentaglycine-modified catheters were deployed $\pm$ 1 cm within the inferior vena cava via the femoral vein in 6–8-week-old C57BL/6 mice. All animals were maintained under anaesthesia with isoflurane or ketamine on a heating pad. To functionally modify catheters in situ, a 200 μl solution containing 4 nmol eSrtA, 40 nmol LPETG-tagged biotin and 20 U of heparin was administered intravenously through the catheter inlet followed by a 100 μl saline flush. To demonstrate in situ removal of LPETG-tagged biotin probes from pentaglycine-modified catheters, a 200 μl solution containing 40 nmol eSrtA, 2 μmol GGG peptide and 20 U of heparin was injected intravenously through the catheter inlet followed by a 100 μl saline flush. Mice were sacrificed 1 h later and catheters were explanted and incubated in streptavidin-Cy3 (30 min) and washed with TBS buffer (2 h). Fluorescent and bright field microscopy images were obtained to detect biotin groups on the catheter surface. Real-time imaging of in situ removal of LPETG and Alexa Fluor 750 was performed using a Maestro Multi-Spectral in vivo fluorescence imaging system with near-IR filter sets. Images were taken using a monochromatic 12-bit camera and analysed using ImageJ to determine fluorescence intensity.

**In situ modification of catheters deployed in rats.** DBCO-activated PU catheters were generated as described above, reacted overnight with NH$_2$-GGGGG-N$_2$ at 37 °C, and rinsed in TBS buffer. Free cyclooctyne groups were blocked with 2-azidoethanol (25 mM) in TBS at RT for 1 h and catheters then rinsed with TBS overnight in the dark. Pentaglycine-capped catheters were subsequently reacted with 10 μM Texas Red-TM-PEG$_2$ and 1 μM eSrtA for 1 h at 37 °C. Under anaesthesia, pentaglycine or Texas Red-TM catheters were deployed within the external jugular vein of Wistar rats (75–100 g, male). The external end of the catheter was trimmed and tacked into the subcutaneous soft tissue and held in place with a suture. Catheters were maintained in vivo without administration of a systemic anti-coagulant or anti-platelet agent. Seven days after catheter implantation, rats were anaesthetized and ‘stripping’ or ‘charging’ reagents were delivered intravenously via the dorsal penile vein (n = 5/group). To charge pentaglycine catheters, 80 nmol Texas Red-TM-PEG$_2$ with or without 8 nmol eSrtA was delivered (400 μl/saline). To strip catheters, 8 μmol GGG with or without 160 nmol eSrtA was delivered (400 μl/saline). For recharging experiments, catheters were stripped in vivo and 24 h later, rats were anaesthetized and charging agents delivered intravenously. Reagents were allowed to circulate for 1 h before catheter removal and imaging by fluorescence microscopy (Axio Zoom, Zeiss). Fluorescent intensity was quanified using ImageJ64. Catheters displayed no signs of adherent thrombus upon removal (Supplementary Fig. 11).

**Statistical Analysis.** Mean and s.d. were calculated for each parameter. Tests for significance between two groups were conducted with the Student’s t-test. Tests among three or more groups were conducted with analysis of variance. Values of P < 0.05 were considered statistically significant.

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Author contributions
H.O.H. and Z.Q. performed experiments and analyzed the data; B.M.D. and W.K. contributed to protein expression; E.D. performed in vivo experiments; C.A.H. designed the TM construct; E.L.C., D.R.L., Z.Q., B.M.D., and C.A.H. wrote the manuscript.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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