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RESEARCH PAPER

Stability of a Therapeutic Layer of Immobilized Recombinant Human Tropoelastin on a Plasma-Activated Coated Surface

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ABSTRACT

Purpose To modify blood-contacting stainless surfaces by covalently coating them with a serum-protease resistant form of tropoelastin (TE). To demonstrate that the modified TE retains an exposed, cell-adhesive C-terminus that persists in the presence of blood plasma proteases.

Methods Recombinant human TE and a point mutant variant (R515A) of TE were labeled with ^{125}Iodine and immobilized on plasma-activated stainless steel (PAC) surfaces. Covalent attachment was confirmed using rigorous detergent washing. As kallikrein and thrombin dominate the serum degradation of tropoelastin, supraphysiological levels of these proteases were incubated with covalently bound TE and R515A, then assayed for protein levels by radioactivity detection. Persistence of the C-terminus was assessed by ELISA.

Results TE was significantly retained covalently on PAC surfaces at 88 ± 5% and 71 ± 5% after treatment with kallikrein and thrombin, respectively. Retention of R515A was 100 ± 1.3% and 87 ± 2.3% after treatment with kallikrein and thrombin, respectively, representing significant improvements over TE. The functionally important C-terminus was cleaved in wild-type TE but retained by R515A.

Conclusions Protein persists in the presence of human kallikrein and thrombin when covalently immobilized on metal substrata. R515A displays enhanced protease resistance and retains the C-terminus presenting a protein interface that is viable for blood-contacting applications.

KEY WORDS kallikrein · plasma-activated · thrombin · tropoelastin

ABBREVIATIONS

^{125}I	iodine-125
316L SS	316L stainless steel
ANOVA	analysis of variance
PAC	plasma-activated coating
PEO	polyethylene oxide
R515A	point-mutant tropoelastin SHELΔ26A(R515A)
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
TE	tropoelastin (SHELΔ26A)

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INTRODUCTION

The covalent immobilization of proteins on materials is increasingly used to construct biosensors (1) and to modulate biological responses to medical implants (2). The types of materials on which this immobilization is desired encompass metals, polymers, ceramics and composite materials. The choice of immobilized molecules is most commonly bioactive proteins. For medical devices, an increasingly popular approach to achieve bio-integration of a medical implant is to adhere surface proteins prior to implantation in order to elicit specific physiological responses.

To maintain the viability of blood-contacting surface proteins, it is essential that the immobilized biomolecule does not degrade and is not quantitatively removed from the surface until it achieves its desired therapeutic effect. An under-explored assumption is that the immobilized therapeutic protein layer is stable following surgical implantation, yet proteins can be degraded by early exposure to endogenous proteases. Blood plasma proteases tend to dominate this early exposure, so plasma-derived proteolysis must be managed if implanted proteins are to functionally persist (3). The activity of plasma enzymes is considerable following the implantation of a biomaterial into the vasculature, caused by activation of the coagulation pathway (4). Significantly up-regulated enzymes are kallikrein and thrombin. Kallikrein is important for activation of the intrinsic pathway of coagulation upon biomaterial surface contact with the blood (5). Thrombin cleaves fibrinogen to form an insoluble fibrin clot that culminates in thrombus formation (4).

Tropoelastin (TE) is a promising candidate to enhance the biocompatibility of substrates for medical applications, as it is a major regulator of vascular cells and thrombogenicity *in vivo* (6,7). However, TE is preferentially cleaved at Arg 515 by diverse serine proteases, including those in human plasma, for example, kallikrein and thrombin (8). In order for or TE to be successfully exploited as a therapeutic protein layer, it would require retention of the C-terminal cell attachment site during blood plasma exposure until cell contact (9–11).

We have covalently immobilized TE to a plasma-activated coating on 316L stainless steel (PAC). The PAC is a surface composed of carbon and nitrogen deposited through a plasma process, which allows binding of proteins through well-characterized free radical species (12). This binding has been shown to resist removal by stringent SDS washing, implying a covalent interaction (13), important for the application of a stable protein coating. The PAC surface is durable and biocompatible (14,15), while covalently immobilized TE increases endothelial cell attachment and proliferation (13). Additionally, the PAC surface and TE-coated PAC exhibit reduced thrombogenicity compared to 316L SS (14), supporting the use of this therapeutic protein coating technology to facilitate the bio-integration of blood-contacting medical devices. Although soluble TE is particularly cleaved by human plasma kallikrein (8), the degradation of covalently immobilized TE has not been explored. It might reasonably be expected that a monolayer of immobilized protein would resist proteolysis through steric hindrance to proteases due to the proximity of a modified PAC surface (13,14,16).

Here we assess whether radiolabeling with ^{125}I can provide a robust method for monitoring the degradation of TE in solution and when immobilized covalently on a PAC surface. Complementary to ELISAs, which detect specific regions of the protein, ^{125}I radiolabeling allows detection of the whole protein. We show that the loss of TE that is immobilized on PAC can be substantially reduced by introducing a single amino acid mutation at Arg 515, chosen specifically to remove a common kallikrein/thrombin cleavage site (8). This persistence correlates with an increased retention of the functional C-terminus of the protein. Increasing the protease resistance of TE is a prerequisite for enhancing the *in vivo* utility of TE-coated PAC surfaces in a blood-contacting environment.

MATERIAL AND METHODS

Materials

Tropoelastin SHELΔ26A (TE) was produced in an *E. coli* expression system and purified as described (17). SHELΔ26A(R515A) (R515A) was produced by small-scale over-expression of the gene construct in *E. coli* using the same methodology (Yeo *et al.* in preparation). The metallic substrate was 25 μm-thick 316L stainless steel foil (316L SS) (Brown Metals).

Synthesis of Plasma-Activated Coating

Plasma-activated coatings on 316L stainless steel were generated from plasma containing a mix of acetylene (10 sccm), argon (4 sccm) and nitrogen (4 sccm). Flow rates of gases were regulated using MKS mass flow controllers. The pressure in the system was maintained at 20 Pa. The pulsed plasma deposition system includes two plasma sources: one radio frequency (RF) electrode at 13.56 Mhz and the other a pulsed DC voltage source. The RF plasma was powered using an ENI-6B RF generator through an ENI Matchwork matching network. The pulsed DC voltage was generated using a RUP-3 pulse generator from GBS-Elektronik (Dresden, Germany). The plasma chamber was grounded to act as an anode electrode. Objects to be coated were placed in electrical contact with the pulsed electrode (18).

^{125}I-Radiolabeling of Protein

Two mg/ml sterile filtered protein in PBS was labeled with ^{125}I using iodination beads (Pierce). One iodobead/mg protein was washed with PBS, dried on filter paper and incubated with 50 μl PBS/bead in a glass vial. One mCi Na^{125}I was added to the beads and agitated every minute for 5 min at room temperature. After incubation, 1 mg protein/bead was added and mixed by agitation every 3 min for 15 min at room temperature. The labeled solution

was desalted through a PD-10 column equilibrated in PBS to remove unreacted $^{125}I^-$. The radioactivity of samples before and after desalting was measured using a scintillation counter (Cobra II Auto-Gamma, Packard BioScience).

Protein Quantification

A BCA assay (Sigma) was used to confirm the presence and concentration of ^{125}I-labeled protein in each fraction to select fractions with the highest concentrations for use in further experiments. Serial dilutions of a 1 mg/ml BSA standard in PBS or samples from each fraction were added to triplicate wells of a 96-well plate, BCA reagent was added to each sample and incubated at 37°C for 30 min, and then the absorbance was measured at 562 nm.

SDS-PAGE Gel Electrophoresis

^{125}I-labeled TE and unlabeled TE were analyzed using a 4–12% gradient SDS-polyacrylamide gel (Invitrogen) and then Coomassie-stained and exposed to CL-Xposure film.

Surface Coating with TE

Stainless steel and PAC samples were incubated overnight at 4°C with 50 μg/ml TE, R515A, ^{125}I-labeled TE or ^{125}I-labeled R515A as determined by the BCA assay. SDS treatment to assess covalent attachment of protein was as described (13).

ELISA

ELISA to detect bound tropoelastin was performed on 0.8×0.6 cm protein-coated samples with or without enzyme digestion. As indicated, samples were washed with SDS, then blocked with 3% BSA. Bound protein was detected with a mouse primary monoclonal anti-elastin antibody BA-4 (Sigma) followed with a goat anti-mouse HRP-conjugated secondary antibody (Sigma). The C-terminal region was detected with a rabbit primary polyclonal anti-tropoelastin C-terminus-specific antibody (kindly provided by Dr. Robert Mecham, St. Louis, USA) followed with a goat anti-rabbit HRP-conjugated secondary antibody (Sigma). Samples were added to ABTS solution and the absorbance measured at 405 nm after incubation for 45 min (13).

Protease Treatment of Immobilized TE and R515A

1.2×0.8 cm pieces of 316L SS or PAC were incubated with ^{125}I-labeled protein overnight at 4°C. Protein-coated samples were incubated with PBS, 10 μM thrombin, 4.5 μM kallikrein (approximately 10 times the physiological concentration of each enzyme (4)) or 0.28 mM human neutrophil elastase (excess) for 16 h at 37°C. Samples were then washed, and the radioactivity was counted using a scintillation counter. The metal samples were then washed with 5% SDS for 10 min at 90°C, washed and counted again. Data were expressed as the percentage of ^{125}I-labeled protein covalently bound after protease exposure compared to the amount of ^{125}I-labeled protein covalently bound to the PAC.

Statistical Analysis

Data were expressed as mean ± SE and indicated in figures as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. Groups were compared by a one-way ANOVA with post-hoc analyses for pair-wise comparisons by Bonferroni post-test. Statistical significance was inferred at a two-sided value of $p<0.05$.

RESULTS

Tropoelastin Mutation to Reduce Protease Susceptibility

The most frequently occurring splice variant of human TE contains 698 amino acids and lacks domain 26A (19). This form of the protein contains characteristic cutting sites for the plasma enzymes thrombin and kallikrein. At physiological concentrations, thrombin preferentially cleaves TE C-terminal to Arg 515 and with less activity at Lys 152, while kallikrein cuts only C-terminal to Arg 515. The Arg 515 protease susceptible site is particularly recognized by human plasma (8). With the aim of reducing the susceptibility of TE to this proteolytic degradation, a mutant form

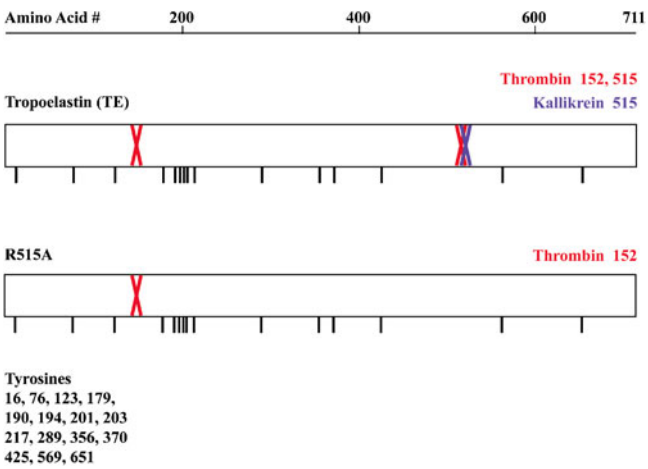

Fig. 1 Schematic of TE and R515A. Representative amino acid sequences of TE and R515A showing possible sites of ^{125}I radiolabeling at tyrosine residues (*black marks*), thrombin cleavage sites (*red*) and kallikrein cleavage sites (*purple*).

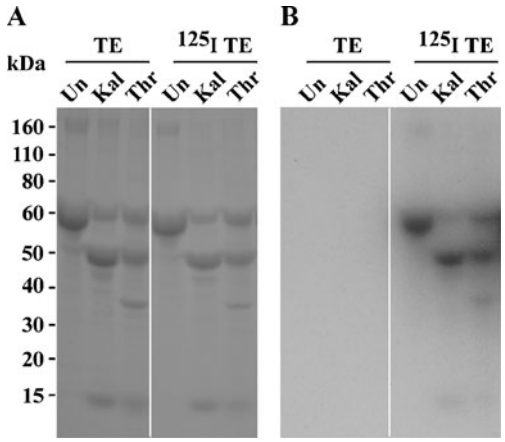

Fig. 2 Verification of ^{125}I labeling and cleavage. TE in solution was either untreated (Un), kallikrein digested (Kal) or thrombin digested (Thr) and analyzed by Coomassie stained SDS-PAGE (**A**). Subsequently, the gel was exposed to X-ray film to visualize the radioactive protein bands (**B**).

of TE was produced with Ala introduced at the 515 position. These two sequences with their proposed protease cleavage sites and candidate sites for Tyr ^{125}I iodination are marked schematically in Fig. 1.

The cleavage of TE by kallikrein and thrombin was confirmed by SDS-PAGE (Fig. 2A). Untreated TE was observed as a 60 kDa species as expected (20). Partial digestion of TE with kallikrein resulted in a lower intensity of the 60 kDa full-length protein band with the appearance of additional bands at 45 kDa and 15 kDa. Thrombin treatment produced fragments of 45, 31 and 13 kDa. The cleavage of TE was unaffected by ^{125}I labeling, as bands of the same mass were observed in corresponding gel digests. Exposure to X-ray film revealed bands corresponding to those observed by Coomassie staining for ^{125}I-TE, but no bands were observed for unlabeled TE. Thus, TE was successfully iodinated, with the label retained on the major cleavage products (Fig. 2B).

Covalent Attachment of TE and R515A to PAC

The binding of TE and R515A to PAC was investigated by detecting the radioactivity of the ^{125}I-labeled proteins and by using an elastin-specific ELISA. Stringent SDS washing was used to remove non-covalently bound protein (12). When incubated with 316L SS, total TE that was physisorbed to the surfaces was detected in the ELISA and radioactivity assay (Fig. 3A and B). Following SDS washing, TE levels returned to background when bound to 316L SS, indicating no covalent attachment. When bound

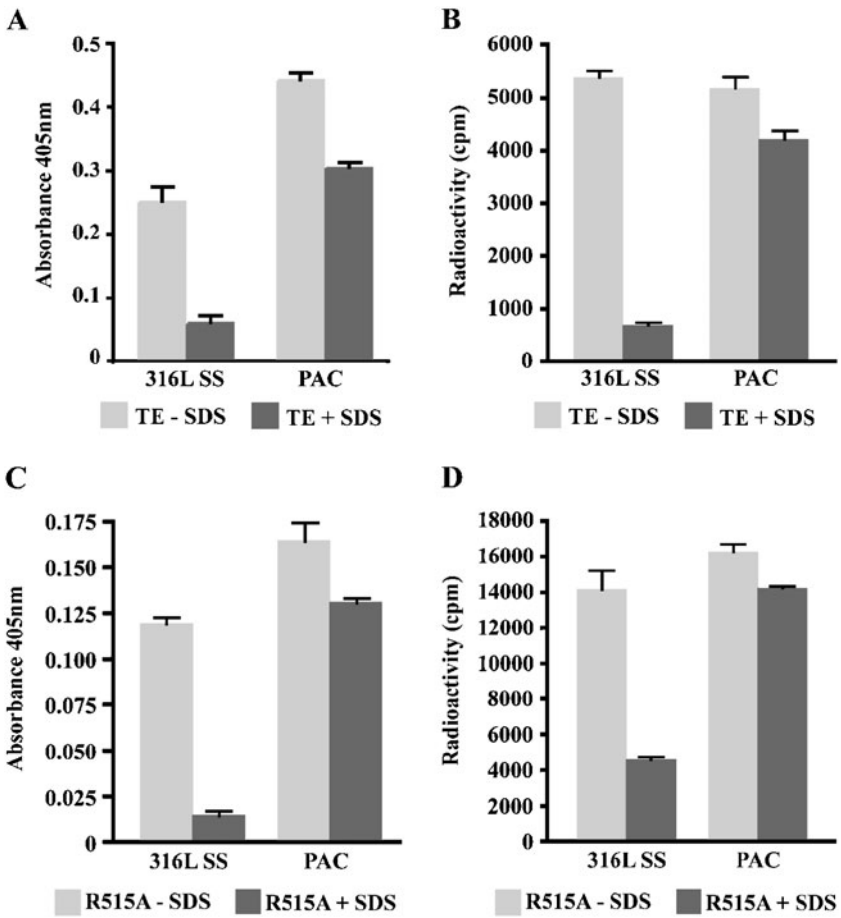

Fig. 3 Covalent binding of TE and R515A to PAC. Binding of (**A**, **B**) TE or (**C**, **D**) R515Ato 316L SS and PAC before (*light grey*) and after (*dark grey*) SDS washing, as detected via an ELISA using the BA-4 primary anti-elastin antibody (**A** and **C**) or ^{125}I radioactivity (**B** and **D**).

to PAC, approximately 71% and 80% TE remained bound after SDS washing for ELISA detection and radiolabel detection, respectively, i.e. TE bound to PAC covalently. Additionally, as the ELISA assay and radiolabel detection correlated, radiolabeling did not interfere with the covalent binding mechanism to PAC. The same trends were observed for R515A (Fig. 3C and D), with 79% and 87% SDS-resistance on the PAC surface as detected by ELISA and radiolabeling, respectively. All conditions contrasted with the lack of binding to a 316L SS control.

Protease Resistance of Immobilized Proteins

To determine the protease resistance of the immobilized TE and R515A, samples of PAC+TE or R515A were incubated with kallikrein and thrombin in excess of physiological concentrations. These effects were compared with extensive digestion with an excess of human neutrophil elastase (Fig. 4). The amount of TE that was covalently retained on PAC was 88±5% ($p<0.05$) and 71±5% ($p<0.001$) after treatment with kallikrein and thrombin, respectively. The amount of R515A retained on PAC was 100±1.3% ($p>0.05$) and 87±2.3% ($p<0.01$) after treatment with kallikrein and thrombin, respectively. All conditions were significantly different from the negative control ($p<0.001$).

C-Terminal Specific ELISA

Kallikrein and thrombin both slightly reduced the amounts of ^{125}I-labeled TE, and only thrombin reduced the amount of ^{125}I R515A that was bound to PAC. Therefore, an

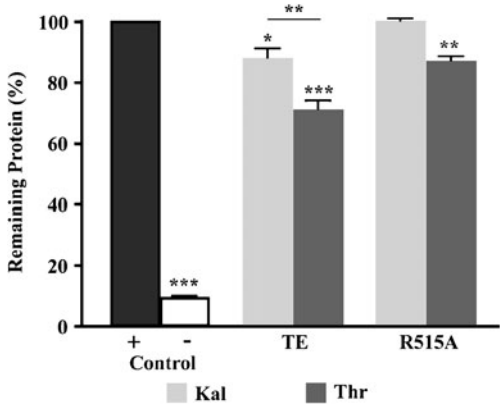

Fig. 4 Percentage loss of covalently bound protein from the PAC after enzymatic degradation. The percentage loss of TE or R515A after enzymatic degradation using a physiological excess of kallikrein (Kal) or thrombin (Thr) was followed by SDS washing. The percentage loss was calculated against the amount of protein covalently bound before enzyme treatment (+ Control). The protease degradation control (− Control) represents the amount of remaining protein after extensive proteolysis of the protein on the PAC surface with human neutrophil elastase.

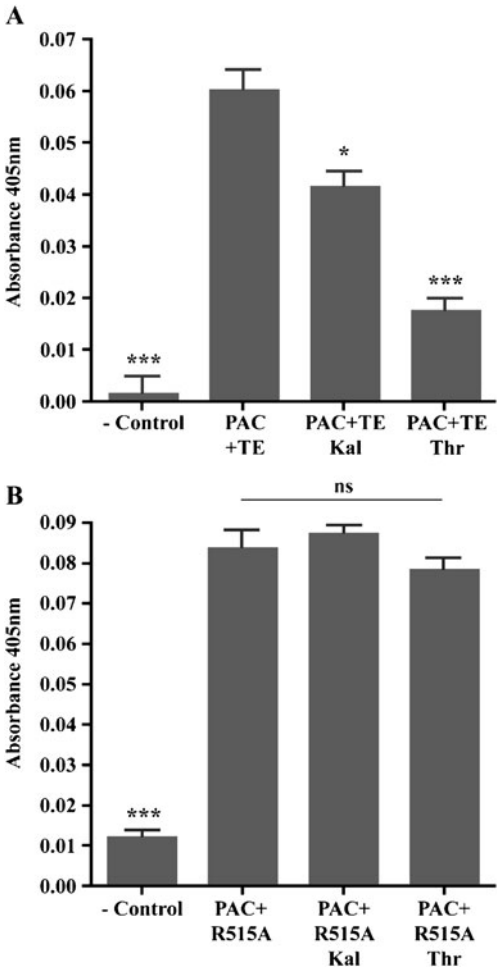

Fig. 5 C-terminal ELISA after enzymatic degradation. ELISA using an antibody specific to the C-terminal region of (**A**) TE and (**B**) R515A after enzymatic degradation with kallikrein (Kal), thrombin (Thr) or no enzyme (PAC + TE or R515A). Negative control samples had no protein (− Control).

ELISA utilizing an antibody specific to a C-terminal sequence of TE and R515A was performed to assess the persistence of the C-terminus after kallikrein and thrombin digestion. After kallikrein and thrombin digestion, there was a significant decrease in the detection of the TE C-terminal region remaining after SDS washing (Fig. 5A). In contrast, no statistical difference in the amount of C-terminal region was detected when R515A was incubated with no enzyme or with the proteases kallikrein and thrombin (Fig. 5B).

DISCUSSION

The increasing application of bioactive protein layers to medical implants is predicated on their resistance to *in vivo* degradation. In blood-contacting applications, breakdown of proteins can be promoted by plasma enzymes that are either basally persistent or activated in response to trauma, including that encountered during the surgical implantation

of devices. TE has been identified as a promising candidate for the bio-functionalization of vascular implants, through its ability to promote endothelialization (13), its ability to inhibit smooth muscle cell growth (6) and its low thrombogenicity (14). However, these studies contemplated less hostile conditions. The stability of TE-coated surfaces after protease degradation has not been studied.

Although many studies have examined the storage stability of immobilized proteins after incubation with saline (21,22), PBS (23) or deionized water (24), relatively few studies have quantified the proteolytic degradation of immobilized protein layers in either *in vivo* or *in vitro* environments. For example, when HRP is immobilized on polyester fabric, it can lose 48% of its activity after incubation with increasing concentrations of trypsin (0.5–2.5 mg/ml) at 37°C for 1 h (25). Furthermore, polyethylene oxide (PEO) with immobilized factor H incubated with serum for 1 h at 37°C shows a decline in factor H availability, but this does not occur in the presence of PBS- or EDTA-containing serum (26). Additional evidence of proteolysis of surface-immobilized protein comes from collagen and sirolimus-coated stents, which rely on surface protein proteolysis to release sirolimus (27).

Our approach was to detect the degradation of covalently immobilized TE and R515A after incubation with supraphysiological concentrations of the major coagulation enzymes, kallikrein and thrombin, by radioactively labeling the protein. Iodination is an accepted methodology to label proteins and quantify the amounts of protein that are adsorbed to surfaces (28–31). Iodination is particularly useful in this instance due to the distribution of tyrosines throughout TE, allowing all possible degradation fragments to be labeled. In combination with ELISAs, this allowed us to detect loss of specific regions of the protein. Using iodinated TE and R515A, we found that these proteins were covalently immobilized on PAC. ELISA analysis using the BA-4 primary elastin antibody correlated with radioactive analyses, revealing 71–80% retention of TE and 79–87% retention of R515A. We have, therefore, demonstrated the ability of the PAC surface to bind covalently to each of the proteins. These findings are consistent with the covalent immobilization of diverse proteins to plasma-mediated coatings and plasma-treated polymers (12). Covalent immobilization is markedly advantageous over simple physisorption because it facilitates an increase in the persistence of protein binding. Furthermore, plasma treatment of surfaces achieves covalent protein binding without the need for chemical linker strategies and thus avoids added steps in the protein attachment process that risk chemically modifying active residues and adding cytotoxicity. Additionally, plasma-treated surfaces are compatible with enhanced conformational stability and maintained activity of bound proteins (32,33). Therefore, this PAC surface offers the opportunity to overcome some of the difficulties associated with the use of intact ECM proteins on synthetic surfaces that require the presentation of a functionally active protein. TE retains its cell binding functionality when bound in this way to PAC surfaces (14).

Our results demonstrate that proteolytic degradation of immobilized TE can be expected to occur by blood contact *in vivo*. This would compromise the effectiveness of the therapeutic protein layer. Modification of the protein at the critical cleavage site R515A mitigates this effect. Full retention of R515A after exposure to supraphysiological kallikrein and majority retention on exposure to thrombin are consistent with the removal of the enzymatic cleavage site at the modified site. This is consistent with C-terminus detection of TE and R515A on PAC. Whereas the amount of C-terminus present on TE-coated PAC was reduced in the presence of kallikrein and thrombin, no loss of C-terminus was observed with R515A-coated PAC. The importance of the C-terminus has been demonstrated to be critical for cell adhesion (10,11). Consistent with its use for bio-functionalization, R515A retains the ability to facilitate C-terminus-dependent cell attachment (Yeo *et al.*, in preparation). Therefore, increased retention of the C-terminus on the PAC surface using the R515A construct offers the opportunity to increase the retention of tropoelastin's biological activity when immobilized on blood-contacting devices.

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