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Cover Letter

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Dear Editor,

In response to the invitation from Dr. Nandita Singh, we are submitting the manuscript titled: “Mitigation of blood borne cell attachment to metal implants through CD47-derived peptide immobilization”. This manuscript describes a new methodology developed to ameliorate platelet and inflammatory cell attachment and activation on the surfaces of stents and other blood-interfacing metallic medical implants. Specifically, through the series of well-characterized robust chemical reactions, we achieved functionalization of the bare metal surfaces with CD47-derived peptide, thus averting immobilization of blood borne cells on the treated surfaces and abrogating downstream pathological responses. Although our therapeutic aims are primarily focused on prevention of in-stent restenosis and thrombosis, this method is of immediate applicability for a wide range of medical purposes, where inflammatory response to biomaterials plays a decisive role. This technology will, therefore, be of interest to research scientists in the fields of biomaterials, bioengineering, and regenerative medicine. Multiple steps of our protocol include elements that are better conveyed to a target audience in visual format rather than as plain textual instructions. Therefore, my co-authors and I think that publication in JoVE can increase the impact of the proposed technology, making it immediately adaptable in a number of laboratories worldwide.

Authors’ contribution

Stanley Stachelek and Ilia Fishbein are responsible for the general study design, development of peptide immobilization techniques, and revising the manuscript.

Vaishali Inamdar and Emmett Fitzpatrick carried out described experiments. Vaishali Inamdar has also drafted the manuscript.

Ivan Alferiev is responsible for the chemical synthesis of the polymers used throughout the described experiments and for the general study design.

Robert Levy contributed to general design, interpretation of obtained experimental results and writing the manuscript.

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With kind regards,

-Ilia Fishbein

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TITLE:

Mitigation of Blood Borne Cell Attachment to Metal Implants Through CD47-Derived Peptide Immobilization

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SUMMARY

Presented here is a protocol for appending peptide CD47 (pepCD47) to metal stents using polybisphosphonate chemistry. Functionalization of metal stents using pepCD47 prevents the attachment and activation of inflammatory cells thus improving their biocompatibility.

ABSTRACT

The key complications associated with bare metal stents and drug eluting stents are in-stent restenosis and late stent thrombosis, respectively. Thus, improving the biocompatibility of metal stents remains a significant challenge. The goal of this protocol is to describe a robust technique of metal surface modification by biologically active peptides to increase biocompatibility of blood contacting medical implants, including endovascular stents. CD47 is an immunological species-specific marker of self and has anti-inflammatory properties. Studies have shown that a 22 amino acid peptide corresponding to the Ig domain of CD47 in the extracellular region (pepCD47), has anti-inflammatory properties like the full-length protein. In vivo studies in rats, and ex vivo studies in rabbit and human blood experimental systems from our lab have demonstrated that pepCD47 immobilization on metals improves their biocompatibility by preventing inflammatory

cell attachment and activation. This paper describes the step-by step protocol for the functionalization of metal surfaces and peptide attachment. The metal surfaces are modified using polyallylamine bisphosphate with latent thiol groups (PABT) followed by deprotection of thiols and amplification of thiol-reactive sites via reaction with polyethyleneimine installed with pyridyldithio groups (PEI-PDT). Finally, pepCD47, incorporating terminal cysteine residues connected to the core peptide sequence through a dual 8-amino-3,6-dioxa-octanoyl spacer, are attached to the metal surface via disulfide bonds. This methodology of peptide attachment to metal surface is efficient and relatively inexpensive and thus can be applied to improve biocompatibility of several metallic biomaterials.

INTRODUCTION

Percutaneous coronary intervention is the first line of therapy to treat coronary artery diseases (CAD) and primarily involves stenting the diseased arteries. However, in-stent restenosis (ISR) and stent thrombosis are common complications associated with stent deployment[1]. Blood interaction at the blood-stent interface is characterized by an almost immediate adsorption of plasma proteins on the metal surface, followed by platelet and inflammatory cell attachment and activation[2]. The release of the inflammatory cytokines and chemokines from activated inflammatory cells leads to the phenotypic modification of the vascular smooth muscle cells (VSMCs) in the tunica media and triggers their centripetal migration to the intimal compartment. Proliferation of activated VSMC in the intima results in intimal layer thickening, lumen narrowing and in-stent restenosis[3]. Drug eluting stents (DES) were developed to prevent VSMC proliferation; however, these drugs have an off-target cytotoxic effect on the endothelial cells[4,5]. Therefore, late stent thrombosis is a common complication associated with DES[6,7]. Stents made of biodegradable polymers, such as poly-L-lactide have shown much promise in the animal experiments and initial clinical trials, but were eventually recalled when the “real-life” clinical use demonstrated their inferiority to the 3rd generation DES[8]. Therefore, there is a need to improve the biocompatibility of bare metal stents for better patient outcomes.

CD47 is a ubiquitously expressed transmembrane protein that inhibits the innate immune response when bound to its cognate receptor Signal Regulatory Protein alpha (SIRPα)[9]. The SIRPα receptor has an immune cell tyrosine inhibitory motif (ITIM) domain and the signaling events upon SIRPα - CD47 interaction ultimately result in the downregulation of inflammatory cell activation[10-13]. Research in our lab has shown that recombinant CD47 or its peptide derivative, corresponding to the 22 amino acid Ig domain of the extracellular region of CD47 (pepCD47), can reduce the host immune response to a range of clinically relevant biomaterials[14-16]. Recently, we have demonstrated that pepCD47 can be immobilized to stainless steel stent surfaces and significantly reduce the pathophysiological response associated with restenosis. Of note, the pepCD47 modified surfaces are amenable to relevant usage conditions such as long term storage and ethylene oxide sterilization[17]. To that end, pepCD47 may be a useful therapeutic target to address the clinical limitations of endovascular stents.

The strategy for the covalent attachment of pepCD47 to a metal surface involves a series of novel chemical modifications of the metal surface. The metal surfaces are first coated with polyallylamine bisphosphonate with latent thiol groups (PABT) followed by the deprotection of

the thiols and attachment of polyethyleneimine (PEI) with installed pyridyldithio groups (PDT). PDT groups of PEI unconsumed in the reaction with deprotected PABT thiols are then reacted with pepCD47 incorporating thiols in the terminal cysteine residues, resulting in binding pepCD47 to the metal surface via a disulfide bond[14,17,18]. We used a fluorophore conjugated pepCD47 (TAMRA-pepCD47) to determine the input concentration of peptide that results in the maximum surface immobilization of the peptide. Finally, we evaluated the acute and chronic anti-inflammatory capacity of the pepCD47 coated metal surfaces, ex vivo, using the Chandler loop apparatus, and monocyte attachment/macrophage expansion assay, respectively.

This paper provides a systematic protocol for the attachment of thiolated peptides to the metal surface; determining the maximum immobilization density of the peptide; and assessing the anti-inflammatory properties of pepCD47 coated metal surfaces exposed to whole blood and isolated monocytes.

PROTOCOL

All human samples for this experiment were obtained in accordance with the IRB of the Children's Hospital of Philadelphia. All animal experiments were performed upon approval from IACUC of the Children's Hospital of Philadelphia.

1. Coating bare metal surfaces with PEI-PDT

1.1. Wash the stainless steel foil coupons (1 cm x 1 cm or 0.65 cm x 1 cm) or stainless steel mesh disks with 2-isopropanol in a shaker (60 °C, speed of 200 rpm) for 5 min. Perform this step 2x. Then wash 2x with chloroform (60 °C, speed of 200 rpm) for 10 min each.

1.2. Place the cleansed stainless-steel samples in an oven at 220 °C for 30 min.

1.3. Prepare 5 mL of 0.5% of PABT solution by dissolving 25 mg of polyallylamine bisphosphonate with latent thiol groups (PABT) and 5 mg of potassium bicarbonate ($KHCO_3$) in 5 mL of DDW and incubate at 72 °C in a shaker at 200 rpm for 30 min.

NOTE: For PABT synthesis refer to previously published literature[18].

1.4. Immerse the baked foils or mesh disks in 0.5% aqueous solution of PABT and incubate in a shaker (72 °C, speed of 200 rpm) for 1 h.

1.5. Wash the PABT-modified samples with deionized distilled water (DDW) for 5x, transfer the specimens in a new vial and wash again with DDW for 5x.

1.6. Prepare a total of 5 mL of TCEP solution (12 mg/mL) by dissolving 60 mg of Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) in 5 mL of 0.1 M acetic buffer (0.57 mL of glacial acetic acid, 820 mg of sodium acetate in 100 mL of DDW).

1.7. Treat the PABT-modified samples with TCEP for 15 min at room temperature (RT) on a shaker.

NOTE: TCEP is used to deprotect the thiol groups.

1.8. Degas DDW in a round bottom flask by using a vacuum generating device, such as a lyophilizer and wash the TCEP treated foils or mesh disks with degassed DDW for 5x. Transfer the samples in a new vial, and additionally wash 5x with degassed DDW.

NOTE: It is paramount to work fast to prevent oxidation of thiols on the metal surface by atmospheric oxygen.

1.9. Prepare 5 mL of 1% PEI-PDT solution by diluting 212.5 µL of stock PEI-PDT and 125 µL of 0.4 M sodium acetate in degassed DDW. Carry out step 1.9 simultaneously with step 1.8 to minimize exposure of the samples to atmospheric air.

NOTE: The synthesis of PEI-PDT is described in previously published literature[18].

1.10. Incubate the washed stainless-steel specimens with 1% PEI-PDT. Replace air with argon gas, seal the vials air-tight and mix on a shaker at RT for 1 h. Either proceed immediately with the peptide conjugation or store at 4 °C up to 1 week.

2. Attachment and qualitative/quantitative assessment of fluorophore conjugated pepCD47 retention on metal surface using fluorescence microscopy and fluorimetry

2.1. Wash foil coupons or mesh disks prepared as described in the section 1, steps 1.1-1.10 above with DDW 5x. Transfer the samples to a new vial and wash with DDW for additional 5x. Finally wash 2x with degassed ethanol and 2x with degassed dimethyl formamide (DMF).

2.2. Prepare tetramethylrhodamine (TAMRA)-conjugated pepCD47 stock solution by dissolving TAMRA-conjugated pepCD47 powder in degassed dimethylformamide (DMF) to a final concentration of 1 mg/mL. Aliquot the stock solution in 1 mL allotments. Store in well-sealed tubes under argon atmosphere at -20 ˚ C.

2.3. Dilute 1 mg/mL of the stock solution of TAMRA-conjugated pepCD47 using degassed DMF to prepare the following concentrations of fluorophore conjugated pepCD47 - 10, 30, 100, and 200 µg/mL.

NOTE: If TAMRA-conjugated pepCD47 appears to be precipitated, reduce the stock TAMRA-conjugated pepCD47 solution using TCEP beads in ratio 1:1, at RT for 20 min. Note that before adding the TAMRA-conjugated pepCD47 to the TCEP beads, spin the TCEP beads solution, remove the supernatant and then proceed with addition of the TAMRA-conjugated pepCD47.

2.4. Incubate the PEI-PDT modified foil coupons with 10, 30, 100 or 200 µg/mL of TAMRA-conjugated pepCD47 in triplicates for each condition on a shaker at RT under argon atmosphere for 1 hour. Incubate PEI-PDT modified mesh disks, as well as mesh disk not modified beyond step 1.2 (bare metal controls) with 100 µg/mL of TAMRA-conjugated pepCD47 in triplicates at RT under argon atmosphere on a shaker for 1 h.

NOTE: From this step onwards, the vials are wrapped in aluminum foil to protect the contents from light.

2.5. Wash the fluorophore conjugated pepCD47-coated surfaces to remove the non-covalently attached peptide in the following order: DMF (3x), DMF/DDW at 1:1, DDW (3x), 0.3% SDS in 20 mM Tris pH 7.4 (3x, 5 min each at 70 °C on a shaker), DDW (3x), change vials, and a final DDW wash.

2.6. Place control and covalently conjugated mesh disks on a microscope glass, add 50 µL of PBS and place a coverslip. Image mesh disks using an inverted fluorescence microscope equipped with a rhodamine filter set. Take representative images at 100x magnification.

2.7. Prepare 15 mL of 12 mg/mL TCEP solution by dissolving 180 mg of TCEP in 1:1 v/v mixture of methanol and 0.1 M acetic buffer.

2.8. Incubate each washed foil with 1 mL of TCEP solution on a shaker at RT for 15 min.

2.9. Prepare the following standards by serially diluting the TAMRA-conjugated pepCD47 stock (1 mg/mL) – 100 µg/mL, 10 µg/mL, 1 µg/mL, 0.1 µg/mL, and 0.01 µg/mL. Use the TCEP solution as the diluent.

2.10. Analyze the TAMRA-conjugated pepCD47 released from the metal surface against the calibration curve generated using the standards by fluorimetry at 544/590 nm excitation and emission wavelengths.

3. Attaching human pepCD47 to PEI-PDT modified surfaces

3.1. Wash PEI-PDT coated samples formulated as described in the section 1, steps 1.1-1.10 above, with degassed DDW 5x, change the vial and wash with degassed DDW 5x.

3.2. Prepare human pepCD47 stock solution (1 mg/mL) by dissolving human pepCD47 powder in degassed 50% acetic acid to achieve a concentration 1 mg/mL.

3.3. Prepare working concentration of human pepCD47 (100 µg/mL) by dissolving 500 µL of the stock of human pepCD47 in 4,500 µL of degassed 1x phosphate buffered saline (PBS).

3.4. Incubate the washed PEI-PDT coated samples with 100 µg/mL of pepCD47 at RT with shaking for 1 h.

3.5. Wash the human pepCD47-coated samples to remove excess peptide in the following order PBS (3x), DDW (3x), 0.2% Tween-20 (3x, 5 min each), DDW (3x), change vials and final DDW wash.

NOTE: Human pepCD47 coated surfaces can be stored dry at 4 °C for up to 6 months.

4. Coating the PEI-PDT modified surfaces with scrambled sequence (Scr)

4.1. Dissolve the scrambled sequence powder in degassed 0.1% acetic acid to prepare a stock solution of 1 mg/mL.

4.2. Prepare a solution of 100 µg/mL of scrambled peptide by dissolving 500 µL of the in 4,500 µL of degassed 0.1% acetic acid.

4.3. Coat the washed PEI-PDT specimens with 100 µg/mL of scrambled peptide at RT with shaking for 1 h.

4.4. To remove unattached scrambled peptide, wash the surfaces in the following order 0.01% acetic acid (3x), DDW (3x), 0.2% Tween-20 (3x, 5 min), DDW (3x), change vials and one DDW wash.

5. Chandler loop for analyzing cellular attachment to metal surfaces

5.1. Coat the metal foils (0.65 cm x 1cm) with either human pepCD47 or scrambled peptide as per the description on section 1 followed by 3 or 4.

5.2. Cut ¼" PVC tubes into three 38 cm long pieces.

5.3. Insert up to 8 unmodified, scrambled peptide or pepCD47 modified metal foils in three different tubes.

5.4. Collect 30 mL of blood from healthy human donors free of any anti-platelet medications as per institutional IRB protocol. Preload syringe with 1 mL of 4% sodium citrate to prevent coagulation of collected blood.

5.5. Put 10 mL of blood into each tube using a 10 mL syringe and connect the ends with metal adapters. Place the blood-filled tubes on the wheels of the Chandler loop apparatus.

5.6. Pass the blood along the metal foils by wheel rotation at 37 ˚C at the speed calculated to produce the shear of 25 dyns/cm^2 for 4 h.

5.7. Drain the blood from the tubes and dispose of the blood according to the IRB requirements.

5.8. Cut the tubes using the scalpel to retrieve the foils from each tube.

5.9. Prepare 2% glutaraldehyde solution by diluting the 10 ml of 4% glutaraldehyde solution using 10 mL of sodium cacodylate buffer with 0.1 M sodium chloride.

5.10. Incubate the foils in 2% glutaraldehyde solution for 15 min and store at 4 ˚C overnight. Before analyzing, wash the metal foils 3x with PBS.

6. Analyzing cellular attachment to metal surfaces using CFDA dye

6.1. Warm 8 mL of PBS in 15 mL tube in a water bath set to 37 ˚C.

6.2. Prepare the CFDA (carboxy-fluorescein diacetate, succinimidyl ester) dye solution as follows - add 90 µL of DMSO to one CFDA dye vial to achieve a stock concentration of 10 mM. Next, prepare the working concentration of 93.75 µM by adding 75 µL of the stock CFDA to 8 mL of warm PBS. Mix by inverting the tubes a few times and cover the tube with aluminium foil.

NOTE: It is advisable to freshly prepare the CFDA dye before every use.

6.3. Incubate each foil with 1 mL of CFDA dye in a 24 well plate. Cover the plate with aluminum foil and incubate the plate at 37 ˚C for 15 min.

6.4. Wash the metal foils 3x with PBS to remove excess dye. Image using the inverted fluorescence microscope.

7. Monocyte attachment and macrophage expansion on the pepCD47-modified and bare metal surfaces

7.1. Collect 10 mL of peripheral blood via the vena cava access during the sacrifice of a 400-450 g male Sprague-Dawley rat. Mix immediately with 1,000 IU of sodium heparin to prevent coagulation.

7.2. Pipette 10 mL of density gradient medium into a 50 mL conical tube. Mix the blood with 5 mL of PBS, and carefully layer diluted blood over the density gradient medium using a Pasteur pipette. Centrifuge in a swinging bucket rotor at 800 *x g* and 18-25 ˚ C for 20 min with minimal settings of acceleration and deceleration.

7.3. Using a Pasteur pipette, collect an opaque layer of buffy coat on the interface between plasma and Ficoll. Dilute buffy coat 1:3 with PBS and centrifuge at 550 *x g* and 4 ˚ C for 10 min to buffy coat cells.

7.4. Re-suspend buffy coat cells in 3 mL of ACK lysis buffer to lyse contaminating erythrocytes. Incubate on ice for 4 min. Add 12 mL of cell separation buffer (CSB; 0.5% BSA, 0.5% FBS, 2 mM EDTA/PBS).

7.5. Centrifuge at 550 *x g* and 4 ˚ C for 10 min. Re-suspend the pellet in 10 mL of CSB.

7.6. Centrifuge at 200 *x g* and 4 ˚ C for 10 min to eliminate platelets. Repeat twice.

7.7. Re-suspend the pellet in 500 µL of CSB. Add 10 µg of each of the following mouse anti-rat antibodies: CD8a (clone OX-8), anti-CD5 (clone OX-19), anti-CD45RA (clone OX-33), and anti-CD6 (clone OX-52).

7.8. Incubate at 4 ˚ C on a vertical tube rotator for 1 h. Add 9.5 mL of CSB. Centrifuge at 300 *x g* and 4 ˚ C for 10 min. Discard supernatant, re-suspend the pellet in 10 mL of CSB and repeat centrifugation.

7.9. Re-suspend the pellet in 500 µL of CSB. Add 150 µL of goat anti-mouse IgG microbeads. Incubate at 4 ˚ C on a vertical tube rotator for 20 min. Add 9.5 mL of CSB. Centrifuge at 300 *x g* and 4 ˚ C for 10 min.

7.10. Discard the supernatant. Re-suspend the pellet in 1 mL of CSB.

7.11. Place a LS column in a magnetic separator. Prime the LS column with 3 mL of CSB. Discard the column throughput. Add 1 mL of re-suspended cell pellet from the step 7.10. Start collecting the throughput. After flow stops, add 5 mL of CSB and keep collecting the column throughput until the flow stops.

7.12. Centrifuge the column throughput (6 mL) containing negatively selected monocytes at 300 *x g* and 4 ˚ C for 10 min. Re-suspend the resulting small pellet in 2 mL of RPMI-1640 medium supplemented with 10% FCS, 1% pen/strep and 100 ng/mL rat macrophage colony stimulating factor (M-CSF).

7.13. Count the monocytes using hemocytometer. Adjust monocyte concentration to 5 x 10^5 cells/mL.

7.14. Add 1 mL volumes of monocyte suspension to the individual wells of a 12 well plate with the bare stainless steel foil samples (N=3) or stainless steel samples modified with rat pepCD47 (N=3) as per 1.1-1.10 and 3.1-3.6.

7.15. Change the medium on days 3 and 5 post-seeding. On day 6 post-seeding wash the cells with PBS and fix with 4% paraformaldehyde at room temperature for 15 min. Wash twice with PBS for 5 min.

7.16. Remove the stainless-steel foils and place them individually into a new 12 well plate. Do not flip the foils.

7.17. Incubate in 0.5% Tween-20/PBS for 15 min to permeabilize the cells. Wash twice with PBS for 5 min.

7.18. Block in 10% goat serum/PBS for 20 min. Aspirate the serum. Do not wash. Add mouse anti-rat CD68 antibody (diluted 1:100 in 1%BSA/PBS). Incubate at room temperature for 1 h. Wash 3x in PBS for 5 min each.

7.19. Add goat anti-mouse IgG Alexa Fluor 546 (diluted 1:200 in 1% BSA/PBS). Incubate in dark at room temperature for 45 min. Wash in PBS for 5 min. Counterstain with 1 µg/mL Hoechst 33342 dye in dark at room temperature for 10 min. Wash 3x in PBS for 5 min each.

7.20. Flip the foils and image using a fluorescent microscope with the inverted optics. Capture images at 200x magnification with blue and red filter settings.

7.21. Count the attached monocytes in the individual images and calculated the group averages and standard deviations.

REPRESENTATIVE RESULTS:

The metal surfaces are rendered thiol-reactive for peptide attachment via a series of chemical modifications, as illustrated in **Figure 1**. PABT incubation followed by PEI-PDT treatment makes the metal surface amenable for peptide attachment. Peptide CD47 (pepCD47) containing cysteine residue at C-terminus joined to the core pepCD47 sequence through a flexible dual AEEAc bridge is covalently attached to the thiol-reactive surfaces via disulfide bonds. Using this protocol, we have demonstrated that pepCD47 remains stably attached to the metal surface for up to six months and can withstand normal physiological shear stress and sterilization procedures[17].

The maximum peptide retention was determined by appending TAMRA-conjugated pepCD47 to the metal surface followed by the extensive washing to eliminate non-covalently bound peptide, cleavage of TAMRA-pepCD47 by reduction of disulfide bridges tethering the peptide to the surface, and analytical quantification using a fluorimetric assay. Specifically, increasing input amounts of TAMRA-conjugated pepD47 (1 mL of 10 - 200 µg/mL solution) were appended to the PEI-PDT coated surfaces and washed several times to remove the non-covalently bound peptide. The concentration of covalently bound TAMRA-conjugated pepCD47 was determined by using a reducing agent TCEP to release the covalently attached peptide and assessing its fluorescence against the standards. The input concentration of 10, 30, 100 and 200 µg/mL demonstrated peptide retention of 28 ± 2, 78 ± 2, 182 ± 14 and 157 ± 25 ng/cm^2 respectively (**Figure 2**). Thus, the maximal immobilization density of pepCD47 on the metal surface was found to be approximately 180 ng/cm^2 which was achieved with an input concentration of 100 µg/mL. The proper immobilization of TAMRA-conjugated pepCD47 on the PABT/PEI-PDT-modified metal surfaces was further corroborated by fluorescence microscopy (**Figure 3**) that demonstrated a

uniform fluorescence emitted from the surface of TAMRA-conjugated pepCD47-treated mesh disks (**Figure 3A**). Only minimal fluorescence was detected on the surface of the control meshes that lacked PABT/PEI-PDT modification (**Figure 3B**), thereby excluding a non-specifically bound TAMRA-conjugated pepCD47 as the main source of fluorescence.

Next, we evaluated the ability of the pepCD47 coated surfaces to prevent acute bloodborne cell attachment as compared to the unmodified and scrambled sequence peptide modified surfaces. The scramble peptide has the same amino acid composition as pepCD47 but in a different order[14,17]. The bloodborne cell attachment was evaluated by rotation of blood from healthy human volunteers across unmodified, scrambled, and pepCD47 modified surfaces in the Chandler loop apparatus followed by washing to remove unattached cells, fixation, and staining with CFDA dye. The surfaces were visualized by fluorescence microscopy. Consistent with our previously published data[14,17] pepCD47 coated surfaces show a drastic reduction in bloodborne cell attachment as compared to the scrambled modified and unmodified controls (**Figure 4**).

To expand on the effects of pepCD47 surface modification on inflammatory cell attachment and proliferation, we used negative immunoselection of rat buffy coat cells to isolate monocytes[19], and cultured the isolated monocytes on bare metal and pepCD47-modified stainless steel foils for 6 days in the presence of M-CSF. The results of this study show a combined 58% attenuation of acute monocyte attachment, their phenotypic conversion to macrophages, and macrophage proliferation on the pepCD47 functionalized surfaces (**Figure 5A**) compared to the bare metal controls (**Figure 5 B,C**).

FIGURE LEGENDS:

Figure 1: Schematic representation of the steps involved in appending pepCD47 to metal surfaces. (**A**) The clean metal samples were baked at 220 °C to oxidize the metal surface. The bisphosphonate groups of the polallylamine bisphosphonate with latent thiol groups (PABT) formed co-ordinate bonds with the metal oxides and coat the metal surfaces to form a functionalized monolayer. The PABT coated metal surfaces were further treated with TCEP for deprotection of the thiol groups. (**B**) The structure of PEI-PDT and symbolic representation. (**C**) The PABT coated and TCEP reduced surfaces were treated with PEI-PDT which amplified the total number of thiol-reactive groups available for attachment of the thiolated peptide. Finally, PEI-PDT coated surfaces were reacted with terminal cysteine groups of pepCD47, and the peptide was attached to the surface via disulfide bonds.

Figure 2: Determining the immobilization density of pepCD47 on metal surface. 1 cm x 1cm metal foils were modified using increasing concentrations (10, 30, 100 and 200 µg/mL) of fluorophore conjugated pepCD47. The excess peptide was removed using several washing steps then treated with 1 mL TCEP solution to cleave the fluorophore conjugated fluorophore. The concentration of the peptide covalently attached to the metal surface was analyzed fluorimetrically using a standard curve prepared with defined concentrations of fluorophore conjugated pepCD47. The immobilization density was represented as ng/cm² of peptide attached to the metal surface. Data is expressed as mean ± SEM and is representative of at least three independent experiments.

Figure 3: Fluorescence microscopy imaging of the stainless-steel surface modified with TAMRA-conjugated pepCD47. Stainless steel mesh disks, consecutively modified with PABT, TCEP, PEI-PDT (**A**) or unmodified (**B**) were reacted with TAMRA-conjugated pepCD47. The properly conjugated and control bare metal meshes were extensively washed and imaged at 100x magnification. The scale bar length is 100 µm.

Figure 4: Evaluating acute anti-inflammatory and anti-thrombotic functions of pepCD47. 0.65 cm x 1cm metal foils were coated with 100 µg/mL of either human pepCD47 or scrambled peptide and exposed to blood in the Chandler loop apparatus. The unbound cells were removed by washing with PBS and the foils were fixed in 2% glutaraldehyde. The unmodified, scrambled modified and human pepCD47 modified surfaces were then incubated with the CFDA dye for 15 mins at 37 °C, washed with PBS and analyzed using a fluorescence microscope.

Figure 5: A prevalence of CD68-positive macrophages on the bare and pepCD47-functionalized metal surfaces. Rat peripheral blood-derived monocytes were isolated by gradient density centrifugation followed by negative immunoselection with magnetic microbeads. 5 x 10^5 monocytes were added into the wells of a 12 well plate with individually placed bare metal foil samples (N=3) or the samples derivatized with rat pepCD47. Macrophage differentiation was stimulated by 100 ng/ml M-CSF. Six days after seeding the cells were fixed, and immunostained with anti-rat CD68 antibody, secondary Alexa Fluor-546 (red) conjugated antibody and counterstained with Hoechst 33342 nuclear dye (blue). Representative images of the bare metal (**A**) and pepCD47-functionalized (**B**) surfaces were captured at 200x magnification and merged. The scale bar length is 100 µm.

DISCUSSION

We demonstrate and describe a relatively novel chemical strategy to append therapeutic peptide moieties to a stainless-steel surface with the overarching goal of reducing the surface's reactivity with inflammatory cells found in blood. The bisphosphonate chemistry described herein involves co-ordinate bond formation between the metal oxides and bisphosphonate groups of PABT. The thickness of polybisphosphonate monolayer formed on the metal surface does not exceed 5 nm[18], and, therefore, is inconsequential for the potential proinflammatory effects of the thick polymer coatings[20]. Deprotection of latent thiol group in the aliphatic side chains of PABT primes the metal surfaces for further chemical modification with thiol-reactive compounds. PEI-PDT is branched polyethyleneimine (average Mw=25,000) in which ~20% of ethyleneimine links are modified with thiol-reactive pyridyldithio groups. Since only a minority of PDT groups in PEI-PDT are consumed in the reaction with the PABT-derived thiols, the surface chemistry after PEI-PDT tethering is changed to thiol-reactive to enable attachment of thiolated peptides[18]. This chemical strategy was developed and widely used in our lab for attachment of several biomolecules, such as recombinant proteins[14], peptides[14,17], and viral gene vectors[18] to the metal surface. Although, for most of our work we have used stainless steel surfaces, PABT can interact with other metal alloys[21] and thus has the potential to improve the biocompatibility and therapeutic potential of a wide range of metallic biomaterials.

Our current methodology indicates that the maximum immobilization density of fluorophore conjugated pepCD47 on metal surfaces is 180 ng/cm^2, which is less as compared to our previously published data[17]. We attribute this discrepancy to the different washing strategies used in both studies. In our current protocol we have used SDS which completely removes non-covalently bound peptides as compared to Tween-20 which was used in our initial studies. However, 180 ng/cm^2 corresponds to approximately 4 x 10^6 molecules/µm^2 of pepCD47. This immobilization density is much higher than the physiological levels of CD47 on cell surfaces which are approximately 390 molecules/µm^2[2,22]. Thus, we predict that stringent washing conditions would not significantly affect the anti-inflammatory properties of pepCD47 modified metal surfaces.

This methodology of attachment of pepCD47 to metal is highly reproducible, however there are several steps in the protocol which need careful attention. Firstly, after coating the metal surface with PABT and reducing it using TCEP, the thiols are prone to oxidation when exposed to air. Thus, it is of utmost importance that the surfaces are always submerged in degassed water. For the same reason the PEI-PDT solution is made in degassed water and argon is added to the vials during the incubation of foils coated with PEI-PDT. Secondly, one must consider the potential for the precipitation of solubilized peptides. PepCD47 has a terminal cysteine residue, as well as cysteine residues in the sequence. Thus, when improperly stored, there is a high possibility that the peptides polymerize and precipitate out of the solution. To address this potential problem, it is recommended that the peptides should be reduced using TCEP beads for 20 min to 1 h prior to incubation with PEI-PDT coated surfaces. TCEP would help to reduce the peptides and increase their solubility in their respective diluent. It is also recommended to displace ambient air with argon in the vials while incubating the PEI-PDT coated samples with thiolated peptide.

If the above-mentioned precautions are followed, the coating technique is reproducible and the only potential limitation for this approach is the commercial non availability of reagents PABT and PEI-PDT.

We used a Chandler loop apparatus to provide ex vivo proof of concept analysis to understand the interaction of pepCD47 modified metal surfaces with blood cells and it has been successfully used by our lab to show the anti-inflammatory properties of pepCD47 coated surfaces[14,15,17,23]. In this study, we used CFDA dye that fluoresces only after entering the cells when the acetate groups of the dye are cleaved by the intracellular esterase. The benefits of this staining procedure are that it stains the anucleated platelets and red blood cells as wells as the nucleated cells such as leukocytes. Thus, utilizing CFDA dye provides an assessment of both acute anti-thrombotic and anti-adhesive properties of pepCD47 coated surfaces. More detailed assessment can be acquired through scanning electron microscopy and/or immunostaining, both of which we detailed previously[24]. Results of the current study validate that the human pepCD47 coated metal surfaces are anti-thrombotic and anti-adhesive as compared to the unmodified and scrambled sequence controls.

To further investigate whether pepCD47-modified surfaces alter growth characteristics of attached inflammatory cells, bare metal stainless steel foils or foils formulated with pepCD47 were exposed to isolated rat monocytes in the presence of syngeneic M-CSF. The cells were

cultured under stationary conditions for 6 days to allow the phenotypic conversion and expansion of macrophages. In accordance with our previous results observed in the different experimental settings [14], a profound decrease of inflammatory cell number on pepCD47 functionalized surfaces was demonstrated in the current study.

Thus, our studies ultimately prove that the novel polybisphosphonate chemistry for coating metals with pepCD47 is an effective way of improving biocompatibility of metal surfaces and can be applied in other biomedical applications, such as artificial joints.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

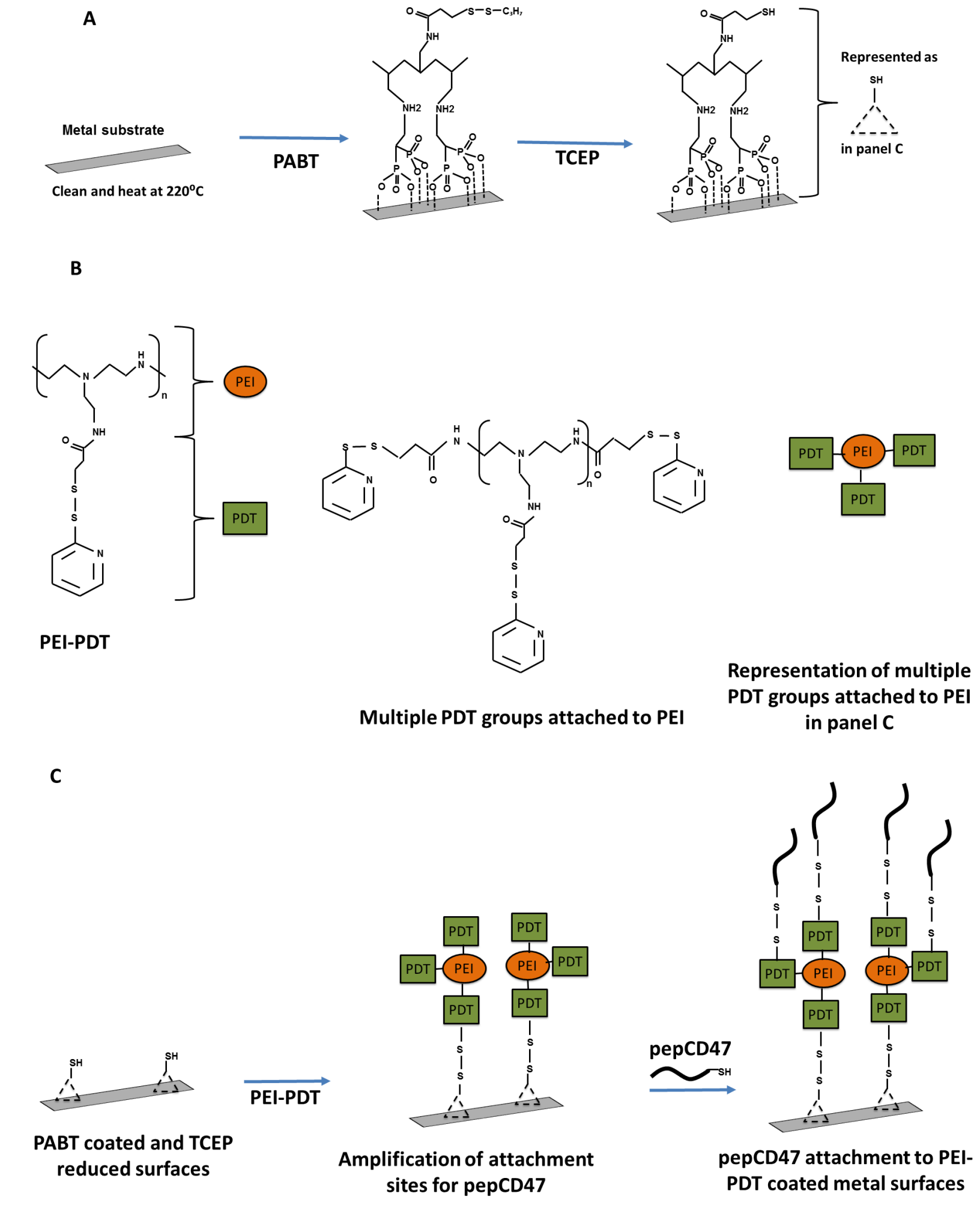

Figure 2

Click here to access/download;Figure;Figure 2.eps

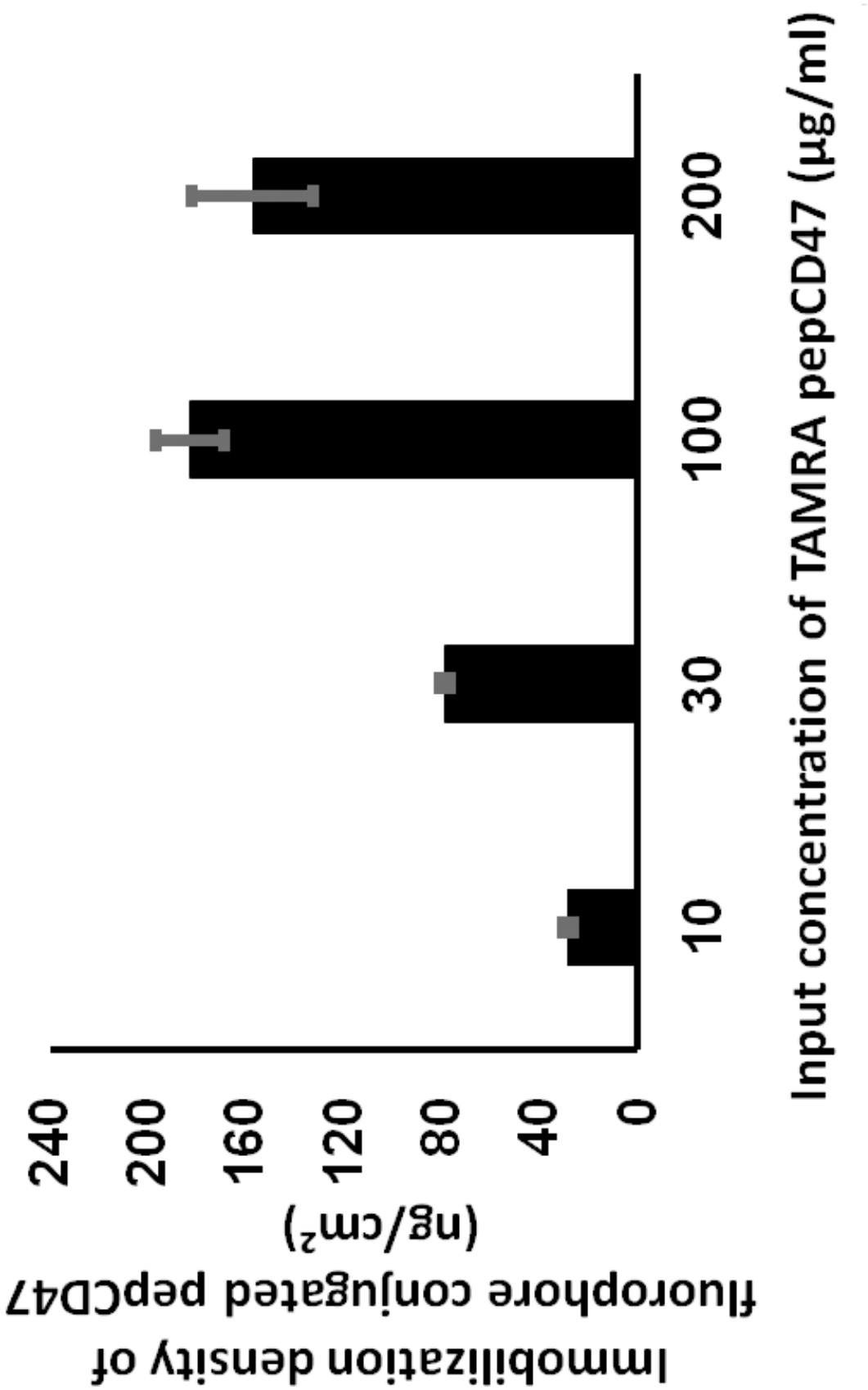

Figure 3

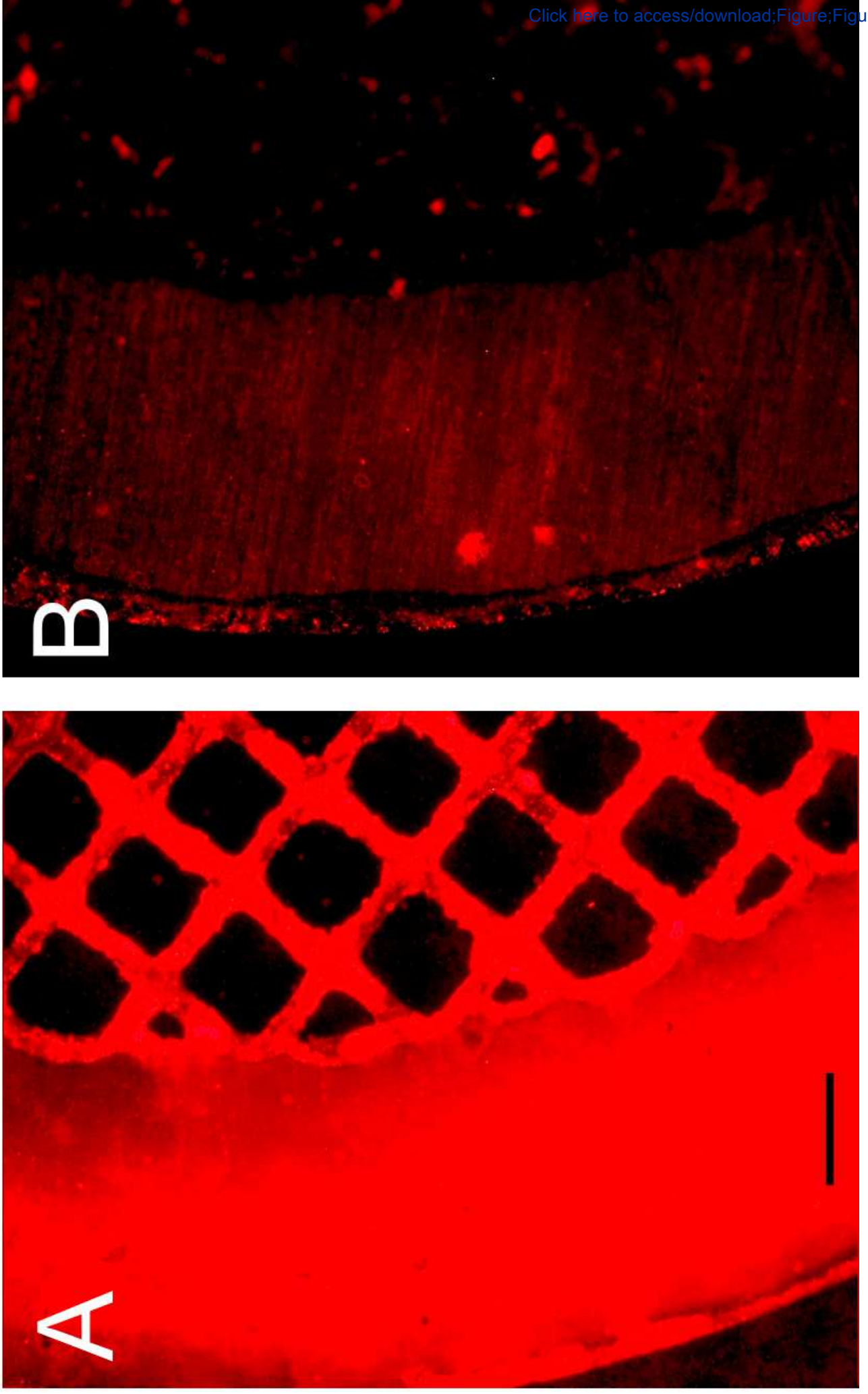

Figure 4

Figure 5

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1 M Tris-HCL	Invitrogen	15567-027	pH - 7.5
4% Glutaraldehyde	Electron Microscopy Sciences	16539-07	
4% Sodium Citrate	Sigma	S5770	
ACK lysing buffer	Quality Biologicals	118-156-721	
anti-CD45RA Ab (mouse anti-rat; clone OX-19)	Biolegend	202301	
anti-CD5 Ab (mouse anti-rat; clone OX-19)	Biolegend	203501	
anti-CD6 Ab (mouse anti-rat; clone OX-52)	BD Biosciences	550979	
anti-CD68 Ab (mouse anti-rat; clone ED-1)	BioRad	MCA341	
anti-CD8a Ab (mouse anti-rat; clone OX-8)	Biolegend	201701	
Chloroform Certified ACS	Fisher Chemical	C298-500	
Dimethyl Formammide (DMF)	Alfa Aesar	39117	
Embra stainless steel grid	Electron Microscopy Sciences	E200-SS	stainless steel mesh mesh disks
Ficoll Hypaque	GE Healthcare	17-1440-02	
Glacial acetic acid	ACROS organic	148930025	
goat anti-mouse IgG Alexa Fluor	ThermoFisher	A11030	
Heparin sodium	Sagent Pharmaceuticals	402-01	
Human pepCD47	Bachem	4099101	
Isopropanol	Fisher Chemical	A426P-4	
Metal adapters	Leur Fitting	6515IND	1 way adapter 316 ss 1/4"-5/16" hoes end
Methanol	RICCA chemical company	4829-32	
Microscope	Nikon Eclipse	TE300	
Phosphate buffered saline (PBS)	Gibco	14190-136	

Pottasium Bicarbonate (KHCO3)	Fisher Chemical	P184-500	
PVC tubes	Terumo-CVS	60050	1/4" X 1/16 8'
sodium cacodylate buffer with 0.1M sodium chloride	Electron Microscopy Sciences	11653	
Sodium Dodecyl Sulfate (SDS)	Bio-Rad laboratories	161-0302	
Sodum actetate (C2H3NaO2)	Alfa Aesar	A13184	
Src peptide	Bachem	4092599	
Stainless steel (AISI 304) cylinder-shaped samples with a lumen	Microgroup, Medway, MA	20097328	1 cm X 6 mm OD
Stainless steel foils (AISI 316L)	Goodfellow, Coraopolis, PA		100 mm X 100 mm X 0.05 mm
Tetramethylrhodamine-conjugated pepCD47 (TAMRA-pepCD47)	Bachem	4100277	
TMB (3,3' ,5,5' -tetramethylbenzidine) substrate and tris (2-carboxyethyl) phosphine hydrochloride (TCEP)	Thermo Scientific	PG82089	
Tween-20	Bio-Rad laboratories	170-6531	
Vybrant CFDA SE Cell Tracer Kit	Invitrogen	V12883	

Dear Editorial staff and reviewers.

The authors are grateful to the editorial office and the reviewers for affirmative and constructive treatment of our manuscript. Most of editing requirements and reviewers' suggestion were incorporated in the revised version.

Please find below a point by point response to the critique.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We proof-read the revised manuscript twice.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

Done.

3. Please define all abbreviations during the first-time use.

All abbreviation were defined as requested.

4. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Presented here is a protocol …"

The short abstract was re-written.

5. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

Our abstract is 240 words long and includes the statement of the protocol goal.

6. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s) without brackets.

Done.

7. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: TAMRA, Chandler Loop, etc.

We completely eliminated commercial language. However, both TAMRA (tetramethylrhodamine) which now is spelled out at the first occurrence in the text, and Chandler loop apparatus are not commercial names, and thus were left in the text of the protocol.

8. Please use generic term throughout.

Done.

9. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.

Done as requested.

10. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

The text in the protocol section is written in the imperative mood.

11. The Protocol should contain only action items that direct the reader to do something.
12. Please ensure you answer the "how" question, i.e., how is the step performed?
13. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

Everything (11-13) was corrected accordingly.

14. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Done.

15. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

All figures are new. No permission from previous publications is required.

16. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Our revised discussion covers all of the suggested topics.

17. Please do not abbreviate the journal titles in the references section.

The official JoVE style in the Endnote use abbreviated journal titles. Nevertheless we changed them for the unabbreviated titles after Endnote formatting.

18. Please sort the materials table in alphabetical order.
Done.

Reviewer 1:

1. In this work, there are many steps of append therapeutic peptide moieties to a stainless steel surface…. could you add some markers to make them visible?
Since the quantification of the immobilized peptide is based on the use of fluorescently tagged CD47, we used fluorescence microscopy to visualize the appended peptide. A new figure (Figure 3) was added to the revised protocol to demonstrate the difference in the surface fluorescence between bare and PABT/PEI-PDT-modified metal surface treated with TAMRA-conjugated pepCD47.

2. … should contain blood compatibility evaluation, such as the proliferation and migration of blood cells on coatings.

3. There is no enough result show the new therapeutic peptide moieties in a stainless steel surface has anti-inflammatory and anti-thrombotic ability, only Figure 3 can't prove the purpose of your work.

In the revised protocol, the authors present a new data (Fig. 5) showing the specific inhibition of macrophage expansion on the pepCD47-modified compared to the bare metal surfaces. Significant reduction in the number of macrophages associated with pepCD47-functionalized surfaces 6 days after the monocyte seeding, reflects both the decreased adhesion of monocytes

and diminished macrophage proliferation and supports the anti-inflammatory effects of pepCD47 coating.

Reviewer 3:

1. The first paragraph is not up to date with the current status of stent bioengineering.

The text was revised according to the reviewer's suggestion.

2. Throughout the paper, the authors should acknowledge that the modified Chandler loops to assess materials can be used to evaluate the acute thrombogenicity and acute inflammatory cell response to materials.

The text was modified to reflect the limitation of the short-term studies performed using the Chandler loop. Additionally, a new Fig. 5 presents the data pertaining to the long-term monocyte culture on the bare metal and pepCD47-modified metal surfaces.

3) What was the blood anticoagulated with? Were the donors screened for recent medication e.g. aspirin?

Blood was anticoagulated with sodium citrate. The donors were free of anti-platelet drugs. The text was modified to convey this information. This information is now included in the revised manuscript.

4) Chandler loops are usually just rotated, so it would be more accurate to say 'Blood was rotated for 4 hours…

The text was corrected as suggested by the reviewer.

5) Error in x axis of Figure 2. Highest dose is 200ug/ml in the paper, but the figure axis shows 300ug/ml.

This error was corrected.