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Hemocompatibility Testing of Blood-Contacting Implants in a Flow Loop Model Mimicking Human Blood Flow

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Manuscript submission

"Hemocompatibility testing of blood- contacting implants according to ISO 10993-4 in a flow loop model mimicking human blood flow"

Dear Dr. Myers,

on behalf of all authors I hereby submit the above-named original research article for consideration for publication in the **Journal of Visualized Experiments (JoVE)**.

The growing use of medical devices for temporary or permanent remain in the bodies circulatory system demands for an increased evaluation of possible complications brought by these devices, such as the activation and destruction of blood components. Thus, in vitro hemocompatibility testing of blood-contacting implants is the first step that paves the way towards its successful in vivo implementation. Therefore, extensive analysis according to ISO 10993-4 is mandatory prior to clinical application.
The presented protocol describes a sensitive model for in vitro hemocompatibility testing of blood-contacting implants in accordance with ISO 10993-4 in a shear flow model imitating human blood flow. The article provides detailed description and explanation for a comprehensive and reliable analysis of hemocompatibility, beginning with blood collection, preparation of the flow-loop model, plasma collection as well as the analysis of blood cell count, the prevalence of several hemocompatibility markers as well as the microscopic visualization of the device surface after blood contact.
Our representative results exemplify how the data generated with the protocol can be assessed and interpreted in a scientific manner. We believe that this manuscript is technically sound and can be an important source of advice and support for other researches.
This manuscript is a single-journal submission and has not been submitted to another journal simultaneously. The submitted manuscript has not been previously published. All authors have read and approved the final version of this paper. The authors report no conflicts of interests.

We would greatly appreciate your consideration of our manuscript for possible publication.

Sincerely,

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TITLE:

Hemocompatibility Testing of Blood-Contacting Implants in a Flow Loop Model Mimicking Human Blood Flow

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KEYWORDS:

hemocompatibility, flow loop model, blood-contacting devices, human blood, ISO 10993-4, evaluation of medical devices

SUMMARY:

This protocol describes a comprehensive hemocompatibility evaluation of blood-contacting devices using laser-cut neurovascular implants. A flow loop model with fresh, heparinized human blood is applied to mimic blood flow. After perfusion, various hematologic markers are analyzed and compared to the values gained directly after blood collection for hemocompatibility evaluation of the tested devices.

ABSTRACT:

The growing use of medical devices (e.g., vascular grafts, stents, and cardiac catheters) for temporary or permanent purposes that remain in the body's circulatory system demands a reliable and multiparametric approach that evaluates the possible hematologic complications caused by these devices (i.e., activation and destruction of blood components). Comprehensive

in vitro hemocompatibility testing of blood-contacting implants is the first step towards successful in vivo implementation. Therefore, extensive analysis according to the International Organization for Standardization 10993-4 (ISO 10993-4) is mandatory prior to clinical application. The presented flow loop describes a sensitive model to analyze the hemostatic performance of stents (in this case, neurovascular) and reveal adverse effects. The use of fresh human whole blood and gentle blood sampling are essential to avoid the preactivation of blood. The blood is perfused through a heparinized tubing containing the test specimen by using a peristaltic pump at a rate of 150 mL/min at 37 °C for 60 min. Before and after perfusion, hematologic markers (i.e., blood cell count, hemoglobin, hematocrit, and plasmatic markers) indicating the activation of leukocytes (polymorphonuclear [PMN]-elastase), platelets (β-thromboglobulin [β-TG]), the coagulation system (thombin-antithrombin III [TAT]), and the complement cascade (SC5b-9) are analyzed. In conclusion, we present an essential and reliable model for extensive hemocompatibility testing of stents and other blood-contacting devices prior to clinical application.

INTRODUCTION:

The in vivo application of implants and biomaterials, which interact with human blood, requires intense preclinical testing focusing on the investigation of various markers of the hemostatic system. The International Organization for Standardization 10993-4 (ISO 10993-4) specifies the central principles for the evaluation of blood-contacting devices (i.e., stents and vascular grafts) and considers the device design, clinical utility, and materials needed[1].

Human blood is a fluid that contains various plasma proteins and cells, including leukocytes (white blood cells [WBCs]), erythrocytes (red blood cells [RBCs]), and platelets, which carry out complex functions in the human body[2]. The direct contact of foreign materials with blood can cause adverse effects, such as activation of the immune or coagulation system, which can lead to inflammation or thrombotic complications and serious issues after implantation[3-5]. Therefore, in vitro hemocompatibility validation offers an opportunity prior to implantation to detect and exclude any hematologic complications that may be induced upon contact of the blood with a foreign surface[6].

The presented flow loop model was established to assess the hemocompatibility of neurovascular stents and similar devices by applying a flow rate of 150 mL/min in tubing (diameter of 3.2 mm) to mimic cerebral flow conditions and artery diameters[2,7]. Besides the need for an optimal in vitro model, the source of blood is an important factor in gaining reliable and unaltered results when analyzing hemocompatibility of a biomaterial[8]. The collected blood should be used immediately after sampling to prevent changes caused by prolonged storage. In general, a gentle collection of blood without stasis using a 21 G needle should be performed to minimize the preactivation of platelets and the coagulation cascade during blood drawing. Furthermore, donor exclusion criteria include those who smoke, are pregnant, are in a poor state of health, or have taken oral contraceptives or painkillers during the previous 14 days.

This study describes an in vitro model for the extensive hemocompatibility testing of stent implants under flow conditions. When comparing uncoated to fibrin-heparin-coated stents,

results of the comprehensive hemocompatibility tests reflect improved hemocompatibility of the fibrin-heparin-coated stents[9]. In contrast, the uncoated stents induce activation of the coagulation cascade, as demonstrated by an increase in thombin-antithrombin III (TAT) concentrations and loss of blood platelet numbers due to the adhesion of platelets to stent surface. Overall, integrating this hemocompatibility model as a preclinical test is recommended to detect any adverse effects on the hemostatic system that are caused by the device.

PROTOCOL:

The blood sampling procedure was approved by the Ethics Committee of the medical faculty at the University of Tuebingen (project identification code: 270/2010BO1). All subjects provided written, informed consent for inclusion before participation.

1. Preparation of heparin-loaded monovettes

1.1. Mix the undiluted heparin (5,000 IU/mL) with sodium chloride (NaCl, 0.9%) solution and prepare a solution with a resulting concentration of 15 IU/mL of heparin.

1.2. Add 900 µL of the diluted heparin solution to each neutral monovette (9 mL) to obtain a final heparin concentration of 1.5 IU/mL after blood sampling. Prepare three monovettes per donor plus three reserve monovettes and store the heparin-loaded monovettes at 4 °C until blood sampling.

2. Blood sampling

2.1. Take the heparin-loaded monovettes out of the refrigerator 30 min prior to blood sampling.

2.2. Collect a 27 mL blood sample from each healthy donor (n = 5) by venipuncture for the flow loop. Only apply a smooth tourniquet to avoid premature activation of the platelets and the blood clotting cascade.

2.3. Collect blood samples in three monovettes containing 900 µL of the heparin solution (1.5 IU/mL) and pool all three monovettes in one plastic container to ensure that all components are evenly distributed.

2.4. Directly transfer the pooled heparinized blood into three different monovettes containing either EDTA (1.2 mL), citrate (1.4 mL), or a mixture of citrate, theophylline, adenosine, and dipyridamole (CTAD, 2.7 mL) to collect baseline values. Proceed with the samples as described in sections 5−8.

NOTE: To guarantee uninfluenced clotting behavior, donors should avoid the intake of hemostasis-affecting drugs (e.g., acetylsalicylic acid, naproxen, and carbenicillin) within the last 14 days, as well as oral contraceptives and smoking.

3. Preparation of the flow loop

3.1. Cut three heparin-coated polyvinyl chloride tubes with a length of 75 cm and inner diameter of 3.2 mm. Load the tubes with the neurovascular laser-cut implants with or without the fibrin-heparin coating. Remember to leave one tub unloaded as a control.

3.2. Place one end of the tube in a reservoir filled with 0.9% NaCl, connect the tubing to the pump head, and insert the other end into a measuring cylinder.

3.3. Adjust the settings of the peristaltic pump to achieve a flow rate of 150 mL/min by using a timer while checking the fill level in the measuring cylinder.

4. Performance of hemocompatibility testing

4.1. Use a 12 mL syringe to fill the tubes with blood. Let 6 mL of blood flow smoothly into each tube containing a sample or unloaded control.

4.2. Form a circuit and close the tubes tightly using a 0.5 cm length of silicone connection tubing. Place the tubes in a water bath of 37 °C and start the perfusion for 60 min.

5. Whole blood count analysis

5.1. Put 1.2 mL of blood after sampling (baseline) or after perfusion into a monovette containing EDTA and carefully invert the tube 5x.

5.2. Insert the monovette into the blood analyzer and perform a blood count analysis for every sample. Then, incubate the monovettes on ice for 15–60 min after the blood count measurement for further analysis, as described in section 7.

6. Collection of citrate plasma

6.1. Fill the monovettes containing citrate with 1.4 mL of blood (freshly drawn or after circulation) and carefully invert 5x.

6.2. Centrifuge the tubes for 18 min at 1,800 x *g* at room temperature (RT). Aliquot three 250 µL samples of the plasma fraction into 1.5 mL reaction tubes and freeze the plasma samples in liquid nitrogen. Store them at -20 °C until analysis.

7. Collection of EDTA plasma

7.1. Incubate the monovettes on ice for 15–60 min after the blood count measurement. Then, centrifuge the tubes for 20 min at 2,500 x *g* and 4 °C.

7.2. Aliquot three 250 µL samples of the plasma fraction into 1.5 mL reaction tubes after

centrifugation and freeze the tubes in liquid nitrogen. Store them at -80 °C until analysis.

8. Collection of CTAD plasma

8.1. Fill the monovettes containing the CTAD mixture with 2.7 mL of blood (freshly drawn or after incubation) and carefully invert 5x. Afterwards, incubate the monovettes on ice for 15−60 min. Then, centrifuge the tubes for 20 min at 2,500 x *g* and 4 °C.

8.2. Transfer 700 µL of the middle plasma fraction into a 1.5 mL reaction tube and centrifuge the filled reaction tubes for 20 min at 2,500 x *g* and 4 °C.

8.3. Aliquot two 100 µL samples of the middle fraction into 1.5 mL reaction tubes after centrifugation and freeze the tubes in liquid nitrogen. Store them at -20 °C until analysis.

NOTE: The collection of EDTA plasma and CTAD plasma can be performed together because the operating conditions are the same.

9. Measurement of human TAT from citrate plasma

9.1. Thaw the citrate plasma in a water bath of 37 °C.

9.2. Use the TAT enzyme-linked Immunosorbent assay (ELISA) kit according to the manufacturer's instructions. Reconstruct the plasma standards and control and dilute the washing solution, anti-human-TAT peroxidase (POD)-conjugated antibody, and chromogen solution. Leave all reagents and the microtiter plate at RT (15−25 °C) for 30 min before starting the test.

9.3. Pipet 50 µL of the sample buffer into each well of the microtiter plate and add 50 µL of the sample buffer (blank), plasma standard, plasma control, and undiluted plasma sample in duplicates to the well plate. Seal the plate and incubate at 37 °C for 15 min with gentle shaking. Then, wash the plate 3x with 300 µL of washing solution.

9.4. Add 100 µL of the POD-conjugated anti-human-TAT antibody to each well. Seal the plate and incubate at 37 °C for 15 min with gentle shaking. Then, wash the plate 3x with 300 µL of washing solution.

9.5. Add 100 µL of the freshly prepared chromogen solution to each well. Seal the plate and incubate at RT for 30 min.

9.6. Remove the seal film and add 100 µL of stop solution to each well. Read the optical density (OD) with a photometer at 490−500 nm. Fit the standard curve data as a trend line and calculate the concentration of the samples.

10. Measurement of PMN-elastase from citrate plasma

10.1. Thaw the citrate plasma in a water bath at 37 °C.

10.2. Use the polymorphonuclear (PMN)-elastase ELISA kit according the to manufacturer's instructions: reconstruct the PMN-elastase control and the PMN-elastase standard to prepare a standard curve using the kit's dilution buffer.

10.3. Dilute the washing solution according to the manufacturer's description. Leave all reagents and the microtiter plate at RT for 30 min before starting the test. Dilute the citrate plasma samples to 1:100 with the dilution buffer.

10.4. Add 100 µL of the sample buffer (blank), PMN-elastase standard curve (15.6−1,000 ng/ mL), PMN-elastase controls (high and low concentrations), and diluted plasma samples in duplicates to the well plate. Seal the plate and incubate at RT for 60 min with gentle shaking. Afterwards, wash the plate 4x with 300 µL of washing solution.

10.5. Add 150 µL of the enzyme-conjugated antibody to each well. Seal the plate and incubate at RT for 60 min with gentle shaking. Afterwards, wash the plate 4x with 300 µL of washing solution.

10.6. Add 200 µL of the 3,3',5,5'-tetramethylbenzidin (TMB)-substrate solution to each well. Seal the plate and incubate at RT for 20 min in the dark. Then, remove the seal film and add 50 µL of the stop solution to each well.

10.7. Read the OD with a photometer at 450 nm with a reference reading at 630 nm. Fit the standard curve data as a trend line and calculate the concentration of the samples.

11. Measurement of terminal complement complex (TCC) from EDTA plasma

11.1. Thaw the EDTA plasma in a water bath at 37 °C, and store on ice after defrosting.

11.2. Use the complement cascade SC5b-9 ELISA kit according to the manufacturer's instructions: dilute the washing solution as described in the manufacturer's protocol. Leave all reagents and the microtiter plate at RT for 30 min before starting the test. Dilute the EDTA plasma samples to 1:10 with the kit's dilution buffer.

11.3. Add 300 µL of washing solution to each well to rehydrate the surface and aspirate after 2 min. Add 100 µL of the sample buffer (blank), SC5b-9 standards, SC5b-9 controls (high and low concentrations), and the diluted plasma samples in duplicates to the well plate.

11.4. Seal the plate and incubate at RT for 60 min. Next, wash the plate 5x with 300 µL of washing solution.

11.5. Add 50 µL of the enzyme-conjugated antibody to each well. Seal the plate and incubate at RT for 30 min. Then, wash the plate 5x with 300 µL of washing solution.

11.6. Add 100 µL of the TMB-substrate solution to each well. Seal the plate and incubate at RT for 15 min in the dark.

11.7. Remove the seal film and add 100 µL of the stop solution to each well. Read the OD with a photometer at 450 nm. Fit the standard curve data as a trend line and calculate the concentration of the samples.

12. Measurement of β-thromboglobulin from CTAD plasma

12.1. Thaw the CTAD plasma in a water bath at 37 °C.

12.2. Use the β-thromboglobulin (β-TG) ELISA kit according to the manufacturer's instructions: reconstruct the β-TG control and the β-TG standard and dilute the washing solution using distilled H_2O. Reconstruct the POD-conjugated antibody using the provided phosphate buffer. Leave all reagents and the microtiter plate at RT for 30 min before starting the test.

12.3. Prepare the standard curve and the control according to the manufacturer's instructions with the provided phosphate buffer. Dilute the CTAD plasma samples to 1:21.

12.4. Add 200 µL of the phosphate buffer (blank), β-TG standards, β-TG controls (high and low concentrations), and diluted plasma samples in duplicates to the well plate. Seal the plate and incubate at RT for 60 min. Afterwards, wash the plate 5x with 300 µL of washing solution.

12.5. Add 200 µL of the enzyme-conjugated antibody to each well. Seal the plate and incubate at RT for 60 min. Afterwards, wash the plate 5x with 300 µL of washing solution.

12.6. Add 200 µL of the TMB-substrate solution to each well. Seal the plate and incubate at RT for 5 min in the dark. Remove the seal film and stop the reaction by adding 50 µL of 1 M sulfuric acid (H_2SO_4) to each well.

12.7. Leave the plate for 15−60 min, then read the OD with a photometer at 450 nm. Fit the standard curve data as a trend line and calculate the concentration of the samples.

13. Sample preparation for scanning electron microscopy

13.1. Remove the implant from the tube using forceps and rinse the implant briefly by dipping it into 0.9% NaCl solution 3x.

13.2. Store in glutaraldehyde solution (2% glutaraldehyde in phosphate-buffered saline [PBS-buffer without Ca^{2+}/Mg^{2+}]) overnight at 4 °C.

13.3. Next, incubate the implants in PBS-buffer for 10 min. Dehydrate the samples by incubating in ethanol with increasing concentration for 10 min each: 40%, 50%, 60%, 70%, 80%, 90%, 96%,

and 100%. Store the dehydrated samples in 100% ethanol until further analysis.

13.4. Perform critical point drying according to the instructions of the drying device or literature[10] just before scanning electron microscopy (SEM).

14. Scanning electron microscopy

14.1. Attach the dried implants to a sample carrier for the scanning microscope and sputter the samples with gold palladium.

14.2. Introduce the sputtered implants into the sample chamber. Take pictures in 100-, 500-, 1,000- and 2,500-fold magnification of the areas with the representative surface and cell adhesion.

REPRESENTATIVE RESULTS:

Briefly summarized, human whole blood was collected in heparin-loaded monovettes then pooled and used to evaluate the baseline levels of cell counts as well as plasmatic hemocompatibility markers.

Subsequently, the tubing containing the neurovascular implant samples was filled, and the blood was perfused for 60 min at 150 mL/min and 37 °C using a peristaltic pump. Again, the number of cells was analyzed in all groups, and the plasma samples were prepared for ELISA analyses (**Figure 1**). The quantification of the blood cells and blood parameters, such as hemoglobin and hematocrit, was performed directly after blood collection as well as after perfusion in the flow loop model for all sample types and the control. No changes were detected regarding the number of WBCs (**Figure 2A**), RBCs (**Figure 2B**), or the hematocrit values (**Figure 2C**). However, a decrease in hemoglobin levels was detected after the incubation of blood in the flow loop model when compared to the baseline values, which was due to the perfusion of blood in the flow loop system (**Figure 2D**). In addition, a decrease in platelet numbers was observed due to blood perfusion. Furthermore, this effect was increased when an uncoated stent was present in the tubing, indicating the adhesion of platelets to the biomaterial. Nonetheless, it was clearly demonstrated that the loss of platelets was significantly higher when the uncoated stent was incubated with blood, as opposed to the fibrin-heparin-coated stent (**Figure 2E**).

Potential alterations of the hematologic plasma markers were also investigated in the test groups after perfusion and compared to the baseline values of the freshly drawn blood. The TAT complex concentration, which reflects the activation status of the coagulation system, was mildly increased due to blood perfusion (**Figure 3A**). In the bare metal stent group, however, a significant increase in the TAT was detected, indicating a profound activation of the coagulation system. The fibrin-heparin-coated stent prevented the activation of the coagulation system, since no increase in the TAT was determined.

The perfusion led to an increased activation of the complement cascade, which was determined by measuring SC5b-9 (**Figure 3B**). However, incubation with uncoated or fibrin-heparin-coated

stents did not further increase the SC5b-9 concentration. Similar results were obtained when analyzing the activation of the neutrophil granulocytes through the quantification of PMN-elastase concentrations (**Figure 3C**).

Visualization of the stent surface was performed using SEM. Clear differences between the two stent groups were detected after blood incubation. While on the surface of the uncoated stent a dense network of blood cells and proteins was present, no adhesion of proteins or cells was detected on the surface of the fibrin-heparin-coated stent (**Figure 4**).

FIGURE LEGENDS:

Figure 1: Schematic overview of the hemocompatibility evaluation of stents in a well-established flow loop model. Fresh human whole blood is collected from healthy donors in blood tubes containing heparin for anticoagulation. For each donor, an empty tube as well as tubes preloaded with the sample material are subsequently filled with fresh blood and incubated in the flow loop at a rate of 150 mL/min at 37 °C for 60 min. Additionally, plasma samples are prepared from freshly drawn blood to obtain the baseline values of each donor. After the incubation, the plasma samples from the test tubing, with and without sample materials, are prepared and analyzed using a specific ELISA.

Figure 2: Analysis of different cell types and blood parameters before and after incubation of different stent implants in the flow loop model. The determination of white blood cells (**A**), red blood cells (**B**), hematocrit (**C**), hemoglobin (**D**), and platelets (**E**) was performed. The data are displayed as mean ± SEM ($n = 5$, $p^* < 0.5$, $p^{***} < 0.001$).

Figure 3: Determination of platelet or immune system activation markers before and after incubation with neurovascular implants. The markers for the (**A**) activation of blood coagulation (TAT), (**B**) complement system (SC5b-9), and (**C**) neutrophils (PMN-elastase) were quantified using ELISA. The analysis was performed on plasma samples gained from freshly drawn blood or blood incubated with different stents in the flow loop model. The data are displayed as mean ± SEM ($n = 5$, $p^* < 0.5$).

Figure 4: Scanning electron microscopic analysis of stents after incubation with blood. The aggregation of blood plasma proteins and platelets on uncoated stent material was observed. In comparison, stent materials with the fibrin-heparin coating did not demonstrate adhesion of cells or other blood components on the surface (magnification of 500-, 1,000-, and 2,500-fold).

DISCUSSION:

The presented protocol describes a comprehensive and reliable method for the hemocompatibility testing of blood-contacting implants in accordance with ISO 10993-4 in a shear flow model imitating human blood flow. This study is based on the testing of laser-cut neurovascular implants but can be performed with a variety of samples. The results demonstrate that this method enables the broad analysis of various parameters such as the blood cell count, prevalence of several hemocompatibility markers, and microscopic visualization of the device surface after blood contact. Using this protocol, potential differences regarding the

hemocompatibility of different devices can be detected.

An alternative to in vitro hemocompatibility assessment consists of in vivo animal testing, which is associated with several disadvantages, such as higher variability and distortion of device-related effects due to the overwhelming short-term effects of tissue injury[6].

For in vitro hemocompatibility testing, three types of models are available: (1) static blood incubation models, (2) agitated blood incubation models, and (3) shear flow models. The static model provides a simple and rapid method to determine thrombogenicity by incubating the device directly with blood, but it only leads to rudimentary results regarding hemocompatibility[11]. To overcome the main disadvantages of static models (i.e., sedimentation of blood cells and the large air-contacting surface), the agitating blood incubation model may be used, in which a test chamber containing the implants is filled with blood and incubated on a rocking platform[12]. However, these model types are still inferior compared to the existing shear flow models, such as the flow loop presented here. The quintessence of these models is that vascular human blood flow can be imitated; thus, a close depiction of the real interaction between the implant and blood cells can be displayed[13]. In addition to the flow loop model, models such as the Chandler loop or several perfused flow chamber exist[14-16].

The Chandler loop is a closed tube system that is partly filled with air and clamped into a rotating device, resulting in blood circulation through the tubing[17]. In the present flow loop system, the tube is completely filled with blood, and the flow is forced by using a peristaltic pump. When using the Chandler loop model, operators face two major disadvantages due to the requirement of including air into the test tubing. First, it is known that the constant interaction of blood and air triggers the aggregation of leukocytes and platelets as well as protein denaturation[18,19]. Second, the blood circulation rate is limited, because the air always remains at the highest point of the loop[20].

These drawbacks can be overcome when using the flow loop system. Since no air-liquid interface is present in the system, no platelet activation occurs. Thus, the model has a low background for thrombotic events so that a low concentration of anticoagulants, typically 1 IU/mL or 1.5 IU/mL of heparin, is sufficient to prevent clotting, even if high flow rates are applied[6]. The adjustable pump-regulated blood flow rate and the freely selectable tube diameter allow the operator to mimic the physiological conditions of a vein or artery, which correspond to the implant to be tested, and achieve relevant test results[21]. However, this advantage is at the same time a limitation, due to the mechanical stress applied to the blood through the pump, and the destruction of erythrocytes (i.e., hemolysis) may occur[2]. This arising intrinsic blood damage reduces the method sensitivity and impedes prolonged exposure to the blood[21]. Nevertheless, several studies have demonstrated the effective use of the flow loop model for hemocompatibility evaluation[22-24].

However, the main gap between all in vitro models and the in vivo mechanisms includes the missing endothelium, which expresses cytokines, anti-thrombotic components, and adhesion molecules; therefore, this component plays a crucial role in the interaction of the implant and

circulating blood[25]. In conclusion, the flow loop model is adjustable, efficient, reliable, and cost-effective to assess the hemocompatibility of implants before clinical use.

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DISCLOSURES:

The authors have nothing to disclose.

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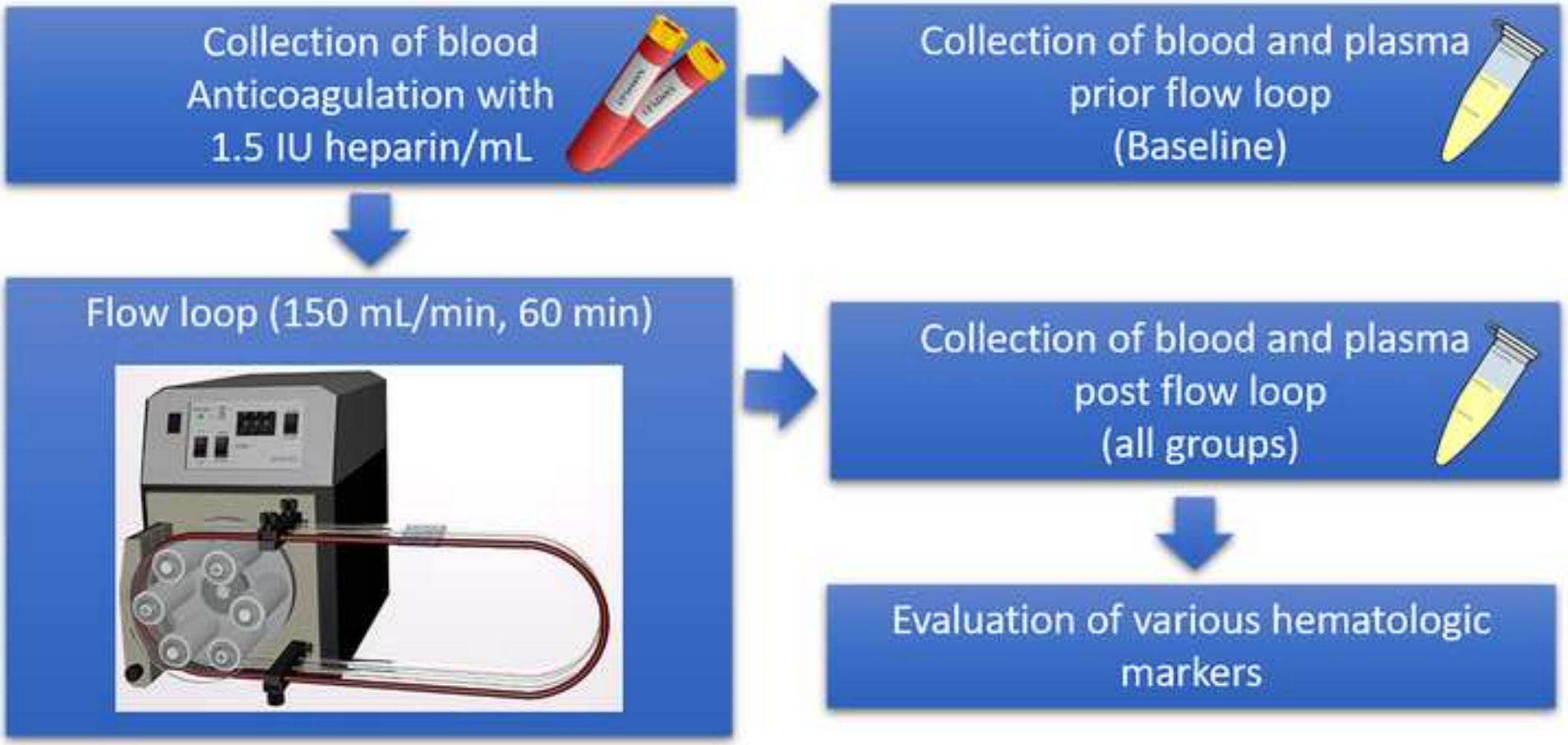
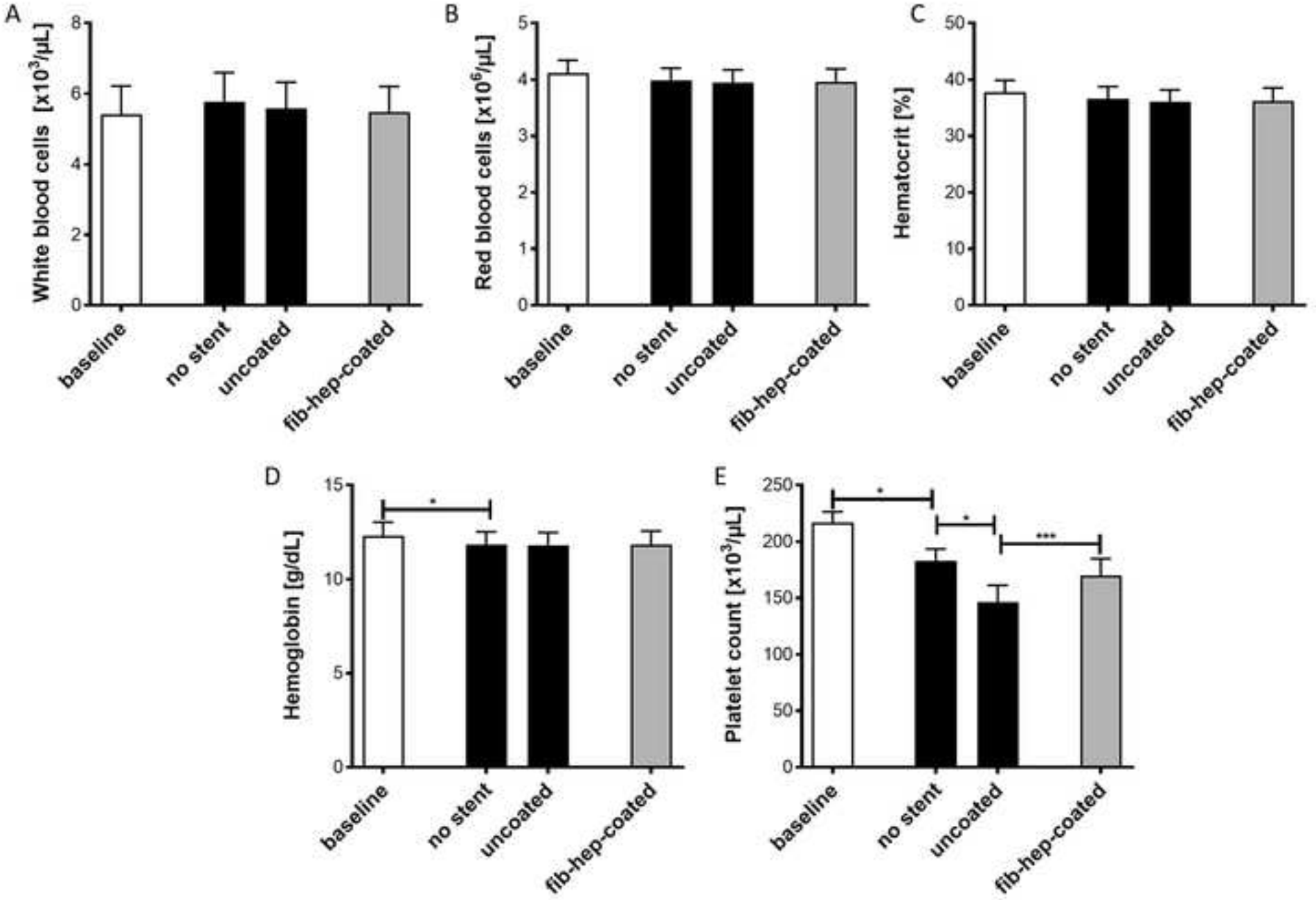
Figure



Figure



Figure

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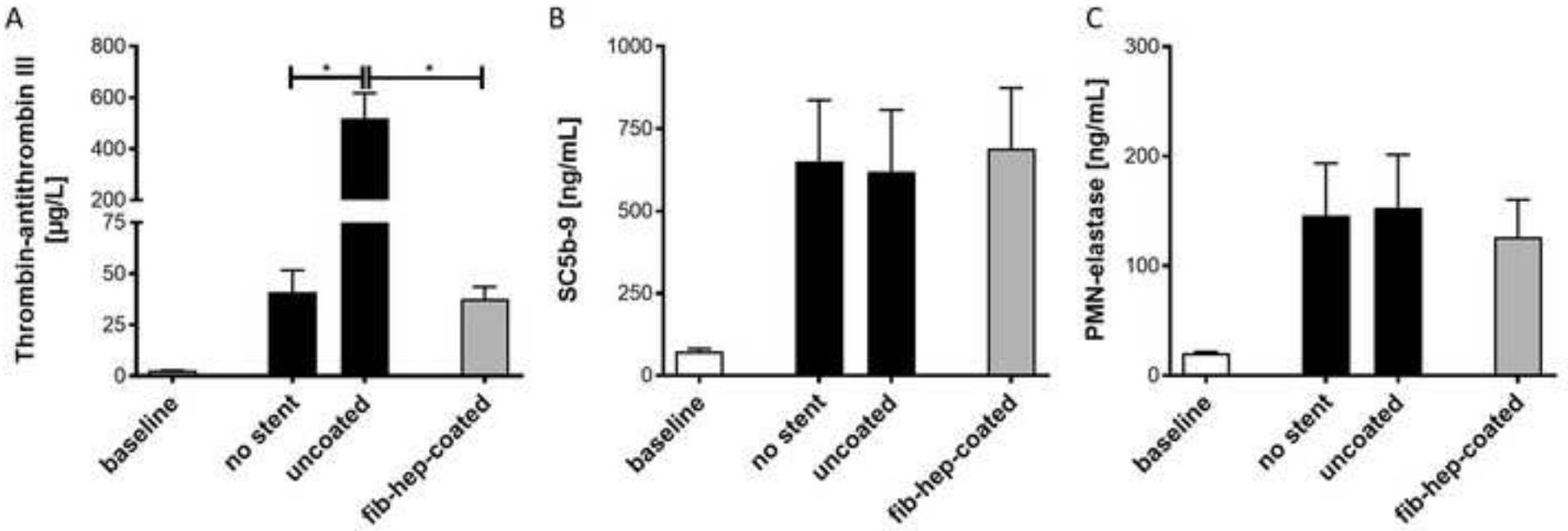

Figure

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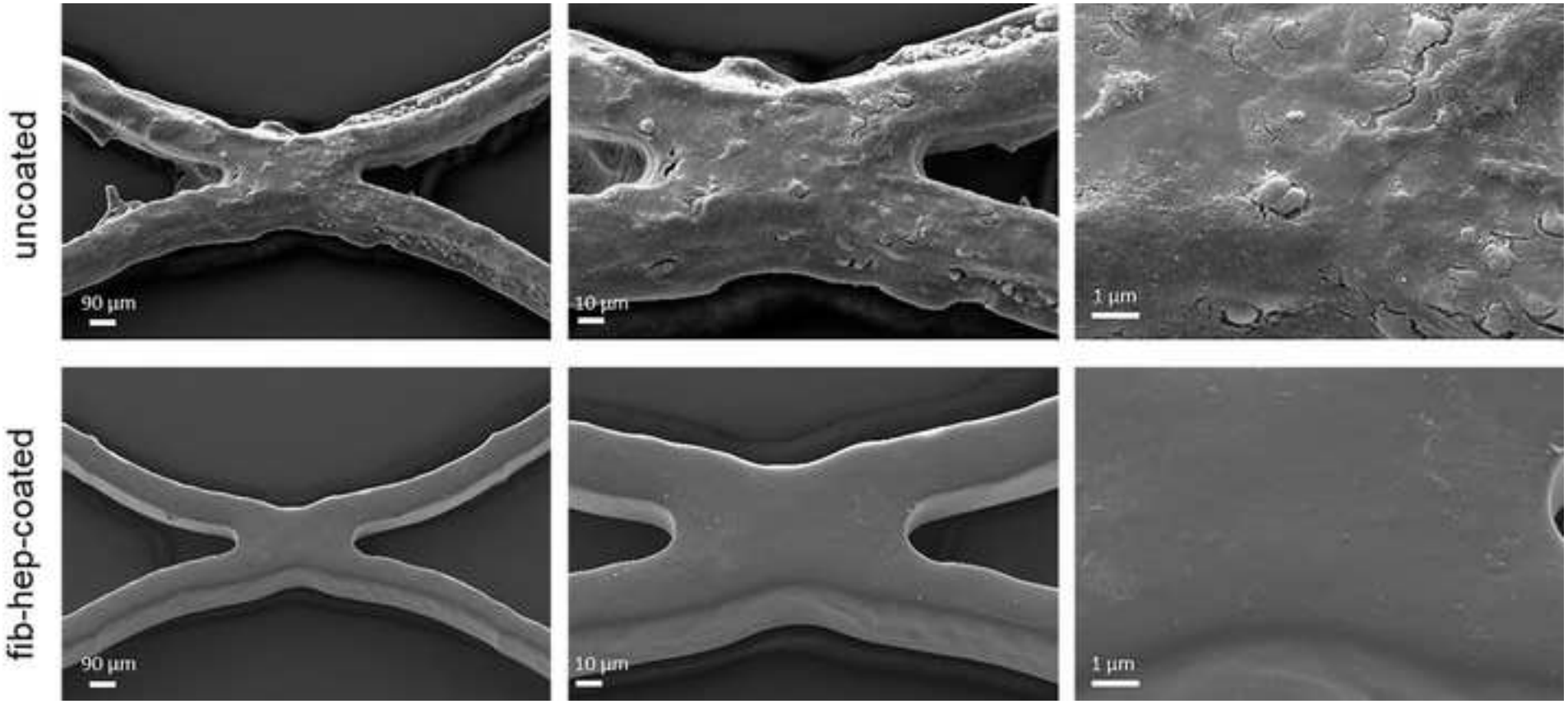

Name of Material/ Equipment	Company	Catalog Number
aqua ad iniectabilia	Fresenius-Kabi, Bad-Homburg, Germany	1088813
beta-TG ELISA	Diagnostica Stago, Duesseldorf, Germany	00950
Centrifuge Rotana 460 R	Andreas Hettich, Tuttlingen, Germany	-
Citrat monovettes (1.4 mL)	Sarstedt, Nümbrecht, Germany	61,668,001
CTAD monovettes (2.7 mL)	BD Biosciences, Heidelberg, Germany	367562
EDTA monovettes (1.2 mL)	Sarstedt, Nümbrecht, Germany	61,662,001
Ethanol p.A. (1000 mL)	AppliChem, Darmstadt, Germany	1,310,861,611
Glutaraldehyde (25 % in water)	SERVA Electrophoresis, Heidelberg, Germany	23114.01
Heparin coating for tubes	Ension, Pittsburgh, USA	-
Heparin-Natrium (25.000 IE/ 5 mL)	LEO Pharma, Neu-Isenburg, Germany	PZN 15261203
Multiplate Reader Mithras LB 940	Berthold, Bad Wildbad, Germany	-
NaCl 0,9%	Fresenius-Kabi, Bad-Homburg, Germany	1312813
Neutral monovettes (9 mL)	Sarstedt, Nümbrecht, Germany	21,063,001
PBS buffer (w/o Ca^{2+}/Mg^{2+})	Thermo Fisher Scientific, Darmstadt, Germany	70011044
Peristaltic pump ISM444B	Cole Parmer, Wertheim, Germany	3475
Pipette (100 µL)	Eppendorf, Wesseling-Berzdorf, Germany	3124000075
Pipette (1000 µL)	Eppendorf, Wesseling-Berzdorf, Germany	3123000063
Plastic container (100 mL)	Sarstedt, Nümbrecht, Germany	75,562,300
PMN-Elastase ELISA	Demeditec Diagnostics, Kiel Germany	DEH3311
Polyvinyl chloride tube	Saint-Gobain Performance Plastics Inc., Courbevoie France	-
Reaction Tubes (1.5 mL)	Eppendorf, Wesseling-Berzdorf, Germany	30123328

neurovascular laser-cut implan	Acandis GmbH, Pforzheim	01-0011x
SC5b-9 ELISA	TECOmedical, Buende, Germany	A029
Scanning electron microscope	Cambridge Instruments, Cambridge, UK	-
Sealing tape (96 well plate)	Thermo Fisher Scientific, Darmstadt, Germany	15036
Syringe 10/12 mL Norm-Ject	Henke-Sass-Wolf, Tuttlingen, Germany	10080010
TAT micro kit	Siemens Healthcare, Marburg, Germany	OWMG15
Waterbath Type 1083	Gesellschaft für Labortechnik, Burgwedel, Germany	-

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Author(s):	Antonia Link, Giorgio Cattaneo, Eduard Brynda, Tomas Riedel, Christian Schlensak, Hans Peter Wendel, Stefanie Krajewski, Tatjana Michel

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Date:	07/29/2019

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We would like to thank the editors and reviewers for their very helpful and constructive comments. We have responded to all the editors' and reviewers' comments on a point-by-point basis.

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1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript, and any errors in the submitted revision may be present in the published version.

Authors' reply: We would like to thank the deputy editor for the opportunity to improve our work. Following the editor's suggestion, we have proofread and corrected the manuscript.

2. Authors and affiliations: Please provide an email address for each author in the manuscript.

Authors' reply: We thank the reviewer for his/her note. The email address and affiliations of each author have been added to the manuscript.

3. Keywords: Please provide at least six keywords or phrases.

Authors' reply: Following your suggestion, we have added six thematically relevant keywords to the manuscript.

4. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.

Authors' reply: Following your suggestion, an ethics statement about the tissue sample being approved by the ethics committee of the medical faculty has been added to the beginning of the protocol.

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Authors' reply: Thank you for the note. We have removed any commercial language from the manuscript.

6. 2.2: What volume of blood sample is collected?

Authors' reply: Thank you for your comment. For the procedure, the total volume of the blood sample is 27 mL per donor. Please see step 2.2.

7. 3.2: Please specify the designated test implants here.

Authors' reply: Thank you for your comment. For the experiment, the following samples were used: neurovascular laser-cut implants with or without a fibrin-heparin coating. This information was added to step 3.1 in the protocol.

8. 6.2, 7.2, etc.: 1.800 x g or 1,800 x g? 2.500 x g or 2,500 x g? Please use the comma symbol (,) for the thousands separator.

Authors' reply: Thank you for your note. The numbers have been corrected in the manuscript, and comma symbols have been used as the thousands separators.

9. Please combine some of the shorter protocol steps so that individual steps contain 2–3 actions and a maximum of 4 sentences per step.

Authors' reply: Sections with short instructions have been combined.

10. Please include single line spacing between each numbered step or note in the protocol.

Authors' reply: The necessary formatting of the single spacing has been adapted according to the requirements.

11. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

Authors' reply: The important parts of protocol have been highlighted.

12. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

Authors' reply: Complete sentences of the protocol have been highlighted in a logical flow.

13. Please include all the relevant details that are required to perform the steps in the highlighting. For example, if step 2.5 is highlighted for filming and the details of how to perform

the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Authors' reply: The relevant details of each step have been highlighted.

14. Please remove the embedded figure(s) from the manuscript.

Authors' reply: Thank you for your suggestion. The figures have been removed from the manuscript and will be submitted separately as graphics.

15. References: Please do not abbreviate journal titles; use full journal names.

Authors' reply: The references section has been revised, and the necessary corrections have been performed.

16. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the protocol. Please sort the materials alphabetically by material name.

Authors' reply: Thank you for the note. We have removed the commercial symbols from the Table of Equipment and Materials.

REVIEWER #1 (Comments to the Author):

Manuscript Summary:

I congratulate the authors for this interesting approach to hemocompatibility sampling and testing. As a result of the research, the answer to their questions presents a very reliable result. Both clinical outcomes and diagnostic data evaluation are often handled without considering the effectiveness of such details.

Major Concerns:

None.

Minor Concerns:

In the discussion part, "Nevertheless, several studies showed effective use of the flow loop model for hemocompatibility evaluation," which referred to references 20, 21, and 22, shared the type of stent that they were using (e.g., silicon-carbide-coated coronary stents, Multilink stent, and hemodialysis membrane, respectively). In this study, the stent type or a brand name were not provided in the method nor materials sections.

Authors' reply: We would like to thank the reviewer for this positive feedback as well as the helpful remark regarding the stents used for the experiments. We have used the following test materials for the experiment: neurovascular laser-cut implants with or without fibrin-heparin coating. We have added them to the protocol as well as to the materials list. However, it was our wish to point out that the test procedure can be used for a variety of products and materials, so we have refrained from mentioning in the title the test material used. Yet, of course, we are ready to add this information if desired by the reviewer.

REVIEWER #2 (Comments to the Author):

Manuscript Summary:

This manuscript describes an approach to in vitro hemocompatibility testing of medical devices intended to be implanted in blood vessels. Specifically, a closed tube, flow loop system is presented with optimized blood collection and blood bioassay procedures.

Major Concerns:

The rationale behind steps in the protocol is missing. For example, in Section 2, blood is collected in three monovettes and then pooled. Why does the blood need to be pooled after collection? Only a smooth tourniquet is called for, and it is unclear why other practices are used during blood draw to promote the premature activation of platelets.

Authors' reply: We would like to thank the reviewer for the feedback and take this opportunity to answer his/her questions in detail. The blood collection tubes have a limited volume of 9 mL each. However, for hemocompatibility testing, a total of 27 mL blood is necessary. After collection, blood pooling ensures that all the blood components are mixed and distributed properly.

It is known that the nativity of the collected blood has a decisive influence on hemocompatibility analysis. Experience demonstrates to us that harsh/long blood stasis causes hemoconcentration, which can lead to incorrectly high levels of proteins, cells, lipids and other analytes, which affect coagulation diagnostics. During blood collection, a 21-gauge needle is used to prevent platelet and coagulation cascade activation.[1] Furthermore, to guarantee an uninfluenced test performance, we only use fresh blood from healthy non-smoking, non-pregnant subjects free of medication.[2]

Several steps rely on commercial assay kits and assume that the reader is familiar with the materials that come in these kits.

Authors' reply: We would like to thank the reviewer for drawing our attention to this. The described analysis is based on the use of the listed assay kits. The descriptions in our protocol are based on the manufacturer's instructions of these kit assays. For professional handling and for safety reasons, we recommend that the reader familiarizes himself/herself with the manufacturer's protocol in advance.

Also, why do the number of wash steps increase with each subsequent assay? Section 9 has the plate washed 3 times between steps, Section 10 has the plate washed 4 times between steps, and Sections 11 and 12 have the plate washed 5 times between steps.

Authors' reply: We would like to thank the reviewer for this comment. However, the manufacturer specifies the number of wash steps in different ELISA kits to guarantee unaltered and reliable results when performing the assay. Therefore, we are not qualified to disregard the manufacturer's statement.

The critical point drying step is important yet as presented, asks the reader to look up the procedure in the literature. It should not be described in this protocol.

Authors' reply: We would like to thank the reviewer for this feedback and fully agree with him/her on this point: the critical point drying is an important step in order to take scanning electron microscopy (SEM) pictures. However, extensively detailed protocols already exist, and their choice strongly depends on the possibilities of the reader since there is a broad range of manual and automatic drying methods and equipment. With this article, we would like to primarily focus on the testing of blood-contacting materials in the flow-loop model and are concerned to go beyond the scope of this article by also presenting a selection of drying protocols. We hope the reviewer understands this.

Minor Concerns:

There are several places in the manuscript where the grammar, word choice, or phrasing is not specific enough and could confuse the reader. For example, the developed setup is intended for devices to be implanted in blood vessels. The phrase "blood-contacting implants" does not capture this fully. What does "double determination" mean? All of the steps with this phrase (9.4, 10.4, 11.5, 12.5) are not clear.

Authors' reply: We would like to thank the reviewer for his/her note. We have changed the protocols to instruct the reader to put each sample in duplicates onto the microtiter plate.

Figure 2D includes only one indicator for statistically significant differences, yet the other two samples look the same as the one that is significant.

Figure 3 includes baseline measurements without an indication of the statistically significant difference between the baseline and the different samples.

Authors' reply: We would like to thank the reviewer for this consideration. The incubation of only blood (no stent) in our flow loop model affects the blood parameters. As you can see in Figures 2 and 3, this incubation leads to PMN-elastase and β-thromboglobulin release as well as a decrease in platelet numbers.

In Figure 2, we demonstrate the significant differences between the baseline and flow loop samples in cell counts. The decrease in cells (WBC, RBC or platelets) indicates the lysis of the cells or aggregation/adhesion of the platelets as an adverse effect of the flow loop or samples. As displayed in Figure 2D, the incubation of the blood in empty tubes has a reducing effect on the percentage of hemoglobin. This effect is a result of the incubation and pumping of the blood in the flow loop model. However, no significant differences between the hemoglobin percentage in the blood samples without or with implant materials after the incubation was detected. To avoid false conclusions, only comparisons between the blood samples with no stents and blood samples with the implants after the incubation were performed, and they revealed no differences.

In Figure 3, the comparison between the baseline and no stent parameters would lead to falsification of the data. Therefore, the baseline is included in the figures to indicated that there is no pre-activation of the blood before incubation in the flow loop model.

Figure 4 includes scale bars with labels that are illegible. Please correct.

Authors' reply: We would like to thank the reviewer for drawing our attention to this. The scale bars as well as the lettering have been improved in the SEM images according to your recommendations.

Organization of the Material /Equipment list to group all equipment together would make finding items, such as the peristaltic pump, easier.

Authors' reply: We would like to thank the reviewer for this comment. The material list and equipment list have been sorted alphabetically according to the editorial board's guidelines.

References

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2 Blok, S. L., Engels, G. E. & van Oeveren, W. In vitro hemocompatibility testing: The importance of fresh blood. *Biointerphases.* **11** (2), 029802, (2016).