Appendix: An In Vitro Blood Flow Loop System for Thrombogenicity Evaluation of Medical Devices and Biomaterials

In vitro blood flow loop thrombogenicity test protocol

1. Introduction

This test protocol is an attachment of the Regulatory Science Tool "An in vitro blood flow loop system for thrombogenicity evaluation of medical devices and biomaterials". This test protocol was adapted from the test methods of the 3 research papers [Refs 1-3] that described the verification studies performed at FDA.

2. Materials & Methods

2.1 Flow loop assembly

The dynamic flow loop consisted of a reusable section of PVC tubing (45 cm long, 6.4 mm ID), that was used for all tests conducted with the same donor blood, inserted into a roller pump and connected to a test section via 6.4 mm OD straight polycarbonate connectors. The test section (**Figure 1A**) of the flow loop was prepared by introducing a 12 cm long test material into the lumen of PVC tubing (32 cm long, 6.4 mm ID), through a small cut in the PVC tubing wall, and the incision site was sealed with Parafilm. One test sample was used in each blood flow loop. Using a multi-channel roller pump, two loops were run simultaneously, and the loops were positioned so that the test sections remained relatively straight (**Figure 1B**). To ensure consistent flow rates within the loops throughout the experiments, the pump tubing sections were preconditioned with phosphate buffered saline (PBS) for 30 min at 70 rpm and the occlusion pressure of each roller pump head was set to 150 mmHg (at 50 rpm) prior to the start of the blood circulation experiments.

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10903 New Hampshire Avenue
Silver Spring, MD 20993
www.fda.gov

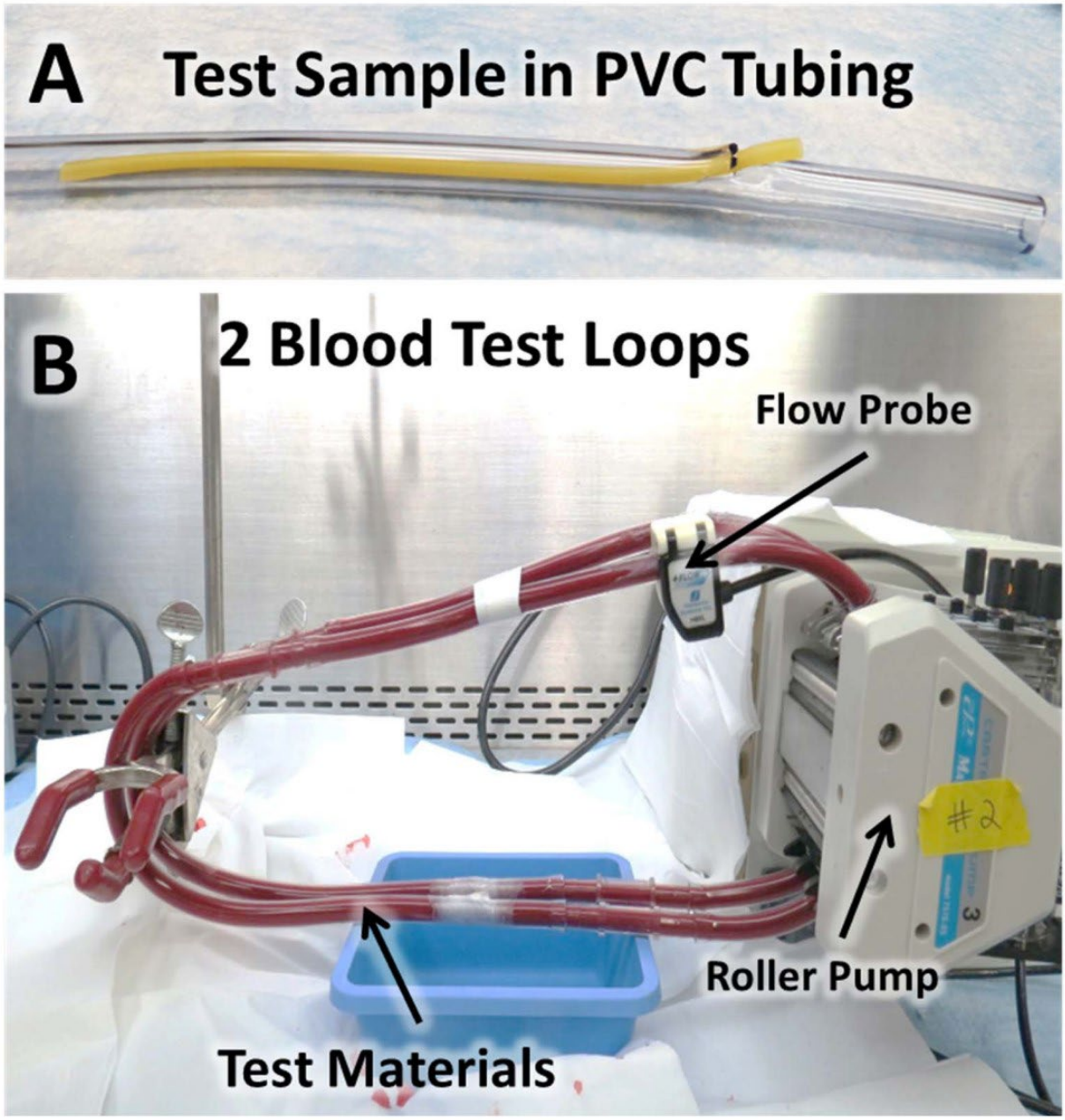

Figure 1: A) Representative image of how a test material is introduced through the sidewall of the PVC tubing into the flow loop. B) Experimental setup of the dual dynamic blood flow loop test systems.

2.2 Test materials

Four to nine test materials with outer diameters of 2.1 – 3.2 mm was used in the verification studies [Ref 1-3]. For the current test system, the use of a negative control polytetrafluoroethylene (PTFE) and a positive control (latex) are necessary to demonstrate the validity and sensitivity of the test. The information for the negative and positive controls is shown in Table 1 below. A comparison device (currently US marketed device with known thrombogenicity profile) should also be tested concurrently with the subject device.

Table 1: Negative and positive materials used in the blood flow loop system.

Test Materials

Material	Outer Diameter (mm)
Latex(Positive Control McMaster Carr Princeton, NJ, Catalog # 5234K931)	3.2
Polytetrafluoroethylene (PTFE) (Negative Control McMaster Carr Princeton, NJ, Catalog # 84935K58)	2.5

2.3 Blood preparation

Human donor blood and four types of animal blood had been used in our verification studies (See **Table 2** below). The human blood (obtained from the National Institutes of Health Blood Bank, Research Donor Program, Bethesda, MD) was drawn into containers with Anticoagulant Citrate Dextrose Solution A (ACDA) from healthy volunteers and used within 6 hrs of the draw. For the animal blood, donor porcine, bovine, and ovine blood (Lampire Biological Laboratories Inc, Pipersville, PA) were drawn into containers with ACDA, shipped to our laboratory overnight, and used within 24-36 hrs of the blood draw. Abattoir porcine blood was collected from a local slaughterhouse into 2.5 U/mL of heparin plus ACDA and used within 12 hrs of the draw.

To remove clots, debris and other foreign contaminants that might have gotten into the blood during blood collection, the abattoir porcine blood was filtered through a 75 µm polypropylene mesh prior to use. For all blood draws, the volume ratio of ACDA to whole blood was 15:85. Blood from three or more unique donors (n=5 or 6 for our verification studies) should be tested separately without pooling. The blood was recalcified prior to circulation in the test flow loop by adding an appropriate amount of CaCl2 solution to the blood to obtain a targeted CaCl2 concentration of 13 mM in whole blood and heparinized to donor-specific heparin concentration as described below.

Table 2: Information about the blood sources used in the dynamic flow loop study

Blood source	Anticoagulant used at blood drawing	Blood transport and storage time before use (hr)	Final heparin concentration for dynamic test (U/mL)
Human	ACDA	2 - 6	1.4 - 2.8
Donor ovine	ACDA	24 - 36	1.4 - 1.8
Donor bovine	ACDA	24 - 36	0.6 - 1.4
Donor porcine	ACDA	24 - 36	3.5 - 7.0

Blood source	Anticoagulant used at blood drawing	Blood transport and storage time before use (hr)	Final heparin concentration for dynamic test (U/mL)
Abattoir porcine	ACDA plus heparin	2 - 12	3.0 - 4.5

2.4 Static pre-test to estimate donor-specific heparin concentration

A static thrombosis-based pre-test was utilized to help determine the donor-specific heparin concentration for individual donors for each blood source. The static pre-test protocol was modified slightly per blood species (ovine, porcine, bovine, human) to account for differences in blood reactivity from different species in response to heparin anticoagulation. Latex tubing sections (4 cm length, 4.8 mm ID, and 8.0 mm OD, Fisher Scientific, catalog #S50616A) were incubated in recalcified blood (8 cm2/mL latex surface area to blood volume) under a series of heparin concentrations (**Figure 2A**) with gentle agitation set at 60 rpm (Edvotek® 10 L Digital Shaking Waterbath, Edvotek Inc., Washington, DC). The samples were incubated for 15 mins at 37°C for all blood species except for donor ovine blood. To reliably target the donor-specific heparin concentration for ovine blood in the dynamic flow loop, the static pre-test incubation conditions had to be adjusted to 30 mins at room temperature. The heparin concentration range was species dependent, as detailed in **Figure 2A**. Based on the preliminary data that correlated thrombus formation from the static pre-test to material performance in the dynamic test, the minimum heparin level that resulted in a thrombus surface coverage ≤ 10% on the latex tubing during the pre-test was defined as the threshold concentration. The target heparin concentration that was selected to initiate the dynamic testing (the “initial concentration”) was based on empirical correlations between the static pre-test and dynamic tests with the positive and negative control materials (as explained below). The differences between the threshold concentrations and the initial heparin concentrations for the flow loop testing were blood species dependent as indicated by the arrows in **Figure 2B-F**.

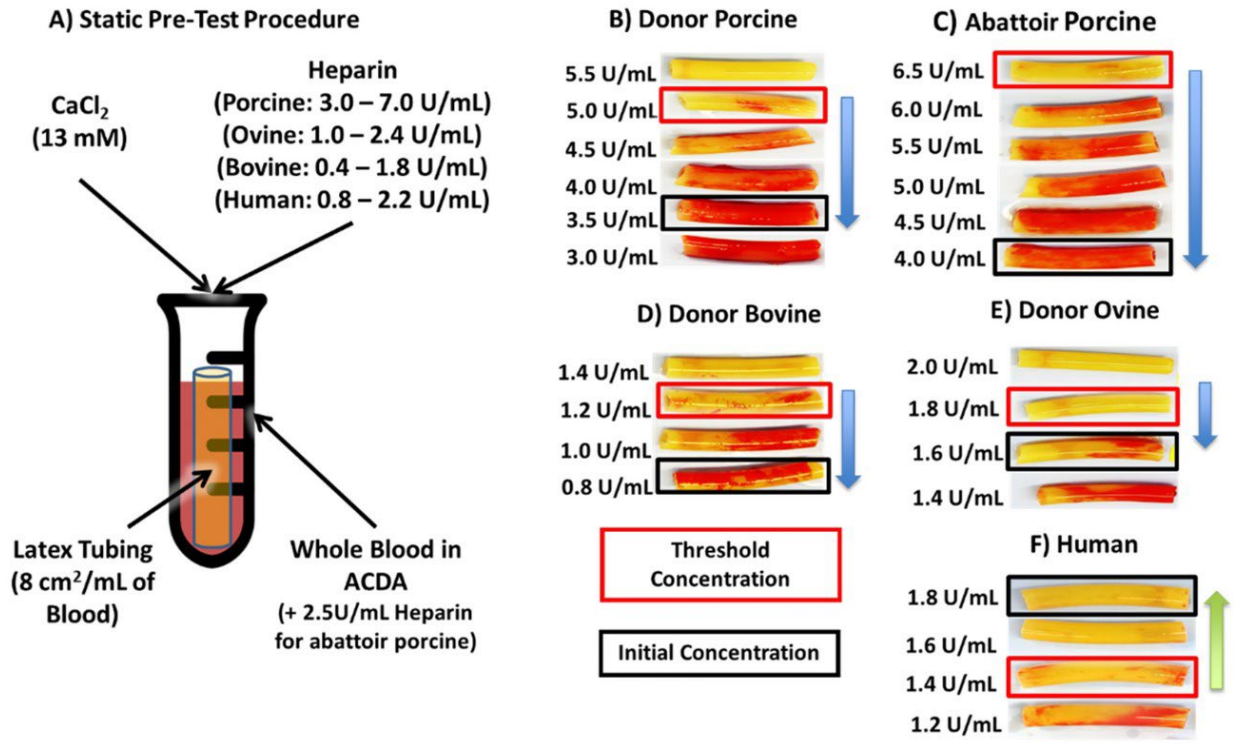

Figure 2: Static latex pre-test performed to estimate donor-specific heparin concentrations. A) Uniform latex tubing segments were incubated in re-calcified blood with a series of species-dependent heparin concentrations for 15 minutes at 37°C (ovine blood was incubated for 30 minutes at room temperature). Example images of the pre-test results: B) Donor porcine blood, C) Abattoir porcine blood, D) Donor bovine blood, E) Donor ovine blood, and F) Fresh human blood. Threshold Concentration is defined as the minimum heparin concentration that resulted in a thrombus surface coverage ≤ 10% on the latex tubing. The difference between the Initial Concentration for the dynamic flow loop test and the Threshold Concentration was blood species dependent, as indicated by the arrows. The initial heparin concentration was 1.5 U/mL lower than the threshold concentration for donor porcine blood, 2.5 U/mL lower for abattoir porcine blood, 0.4 U/mL lower for bovine blood, 0.2 U/mL lower for ovine blood, but 0.4 U/mL higher for fresh human blood.

2.5 Pilot Dynamic test to verify donor-specific heparin concentration

To verify whether the initial heparin concentration selected from the static latex pre-test described in Section 2.4 above would provide appropriate anticoagulation for each blood pool, a set of control materials (negative control: PTFE, positive control: latex) were first investigated using the dynamic flow loop test described in Section 2.6 below. If the reduction in platelet count in the negative control loop was < 30%, and the thrombus surface coverage produced was <10% on the PTFE material and > 50% on the latex materiel (classified as severe thrombus according to NAVI scoring scheme B in Table C.2 of the ISO 10993-4:2017 standard), the initial heparin concentration was used for the remainder of the experiment for that specific donor blood on that day. Although the NAVI scoring scheme categorizes 1% to 25% thrombus surface coverage as minimal thrombus, a lower thrombus surface coverage threshold (10%) on the negative control was used for this study to reduce the variance of the test and to allow for a larger percentage differentiation between the positive and negative control materials. Similarly, the platelet count reduction limit of < 30% in the negative control loop was selected to reduce test variability and improve the differentiation between the positive and negative controls. If the thrombus deposition was > 10% on the PTFE material or < 50% on the latex, or the platelet count reduction was > 30% in the negative control loop, the heparin concentration was increased or decreased (by 0.2 U/mL for ovine, bovine, and human blood or 0.5 U/mL for porcine blood) and this verification process was repeated until the above thrombus surface coverage and platelet count criteria is met. Once an acceptable heparin concentration was determined, the remaining test materials were investigated using that concentration. If an acceptable heparin concentration could not be identified, the blood was discarded and new blood was obtained for replacement.

2.6 Blood Circulation

Whole blood (26 ml) was added to the flow loop and circulated at room temperature (21 ± 2 °C) at a flow rate of 200 mL/min (65-70 rpm) for each of the test samples (**Figure 1B**). This flow rate was selected to produce a physiological venous shear rate of approximately 130 s^{-1}. After 1 hr circulation, the blood in the loops was drained blood and collected for platelet count as described in Section 2.7 below. The loops were then gently rinsed with PBS at a reduced pump speed of 50 rpm to avoid thrombus dislodgement from the test samples. The PVC tubing test sections were removed from the loops and dissected with surgical scissors. The test samples were then carefully removed from the tubing, photographed, and visually inspected for thrombus deposition. The samples were evaluated for surface thrombus coverage and thrombus weight, as described in Section 2.7 below.

Before starting the next test, the reusable pump-head tubing sections of the loops were vigorously rinsed by recirculating PBS for around three min at the highest rpm setting of

the pump (flow rate of approximately 2 L/min). The loops were then drained, and a swab was used to remove any visible thrombi that were attached to the inner lumen of the PVC tubing, before performing a final rinse with PBS.

2.7 Data collection and statistical analysis

To characterize thrombogenicity of the test samples, the percent thrombus surface coverage, thrombus weight, and platelet count reduction were measured. To obtain the thrombus-covered surface areas of the test samples, the lengths of the thrombi were measured using a ruler and their widths were determined by estimating the fraction of the material's circumference covered by the thrombi. Then the percent thrombus surface coverage was calculated by the following equation:

$$\%\ \text{Thrombus surface coverage} = \frac{\sum Thrombus\ Area}{Test\ Material\ Surface\ Area}\ x\ 100\ \%$$

To estimate the thrombus weight after the dynamic flow loop testing, the test materials were dried overnight (≥ 12 hrs at room temperature) and their weights were measured using an analytical balance (Mettler Toledo, XS64 Analytical Balance). The original test material weight, that was collected prior to insertion in the flow loop, was subtracted from the post-test measurement to calculate the dry weight of the adherent thrombi. To account for the diameter difference in the test materials, the dry thrombus weight was then divided by the blood contacting surface area of the test material to obtain a normalized value (mg/cm2).

Blood platelet counts were measured before and after the 1 hr circulation using a complete blood cell counter (Hemavet 950 FS, Drew Scientific Inc., FL) to determine the extent of platelet count reduction during the test.

The data were statistically analyzed using a Friedman repeated measures analysis of variance on ranks with a Newman–Keuls post hoc test. The results were considered statistically significant if $p < 0.05$.

For the current test system, a medical device or biomaterial would be considered passing the test if all three thrombogenicity markers for the test article are not significantly different from those for a comparison device (currently US marketed device with known thrombogenicity profile) or the negative control, and are significantly less thrombogenic than the positive control. The test article would be considered failing the test if all thrombogenicity markers are not significantly different from those for the positive control, or all markers are significantly greater for the test article relative to the

comparison device. For situations between the above pass and fail scenarios, the thrombogenic risks should be evaluated case-by-case and additional testing may be needed.

References:

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