REPORT

TEST FACILITY

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CONFIDENTIAL

STUDY TITLE

ASTM Hemolysis Study

TEST ARTICLE NAME

Poly-ond (R) Plating

TEST ARTICLE IDENTIFICATION

Test Panels

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Summary

The test article, Poly-ond (R) Plating, was evaluated for the potential to cause hemolysis. This study was conducted based on ASTM F756, Standard Practice for Assessment of Hemolytic Properties of Materials and ISO 10993-4, Biological evaluation of medical devices - Part 4: Selection of tests for interactions with blood. Anticoagulated whole rabbit blood was pooled, diluted, and added to glassware with the test article in calcium and magnesium-free phosphate buffered saline (CMF-PBS) and in polystyrene tubes with a CMF-PBS test article extract. Negative controls, positive controls, and blanks were prepared in a similar manner. Following incubation for at least 3 hours at 37°C, the samples were centrifuged, and each supernatant collected. The supernatant was mixed with Drabkin's reagent and the resulting solution was analyzed using a spectrophotometer at a wavelength of 540 nm.

The hemolytic index for the test article in direct contact with blood was 8.1% and the hemolytic index for the test article extract was 1.0%. The test article in direct contact with blood was hemolytic and the test article extract was non-hemolytic.

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1. Introduction

1.1 Purpose

The purpose of this study was to evaluate the potential to cause hemolysis. Both the test article and an extract of the test article were tested *in vitro*.

1.2 Testing Guidelines

This study was conducted based on the ASTM F756, Standard Practice for Assessment of Hemolytic Properties of Materials and the ISO 10993-4, Biological evaluation of medical devices - Part 4: Selection of tests for interactions with blood.

This test was performed under an ISO 13485 certified Quality System, with the test method accredited to the ISO 17025 Standard.

1.3 Dates

Test Article Received:	April 28, 2017
Test Conducted:	May 19, 2017

2. Identification of Test and Control Articles

The test article provided by the sponsor was identified and handled as described below:

Table 1: Test Article

Name:	Poly-ond (R) Plating
Identification:	Test Panels
Physical Description of the Test Article:	1x1x.032 Steel test panels and 1/16 diameter X 3/8 test rods. Both test samples were plated with our Poly-ond Nickel Teflon coating.
Storage Conditions:	Room Temperature

Table 2: Negative Control Article

Name:	High density polyethylene (HDPE)
Strength, Purity, Composition or Other Characteristics:	Composition: polyethylene

Table 3: Positive Control Article

Name:	Sterile Water for Injection (SWFI)
Strength, Purity, Composition or Other Characteristics:	Purity: Meets requirements of USP <643> Total Organic Carbon and USP <645> Water Conductivity Grade and USP <85> Bacterial Endotoxin testing and is certified as USP grade; Composition: Neat CAS No.: 7732-18-5

Table 4: Vehicle

Name:	Calcium and magnesium-free phosphate buffered saline (CMF-PBS). CMF-PBS composition: KH_2PO_4 = 144 mg/L, NaCl = 9,000 mg/L, Na_2HPO_4 = 795 mg/L.

3. Test System

3.1 Test System

Whole blood samples collected from rabbits into vacuum tubes containing EDTA as the anticoagulant were maintained at room temperature and used within five hours of collection.

Breed:	New Zealand White
Source:	Robinson Services, Inc.
Sex:	Female
Number of Donors:	Three
Estimated Date of Birth:	December 17, 2016 and December 31, 2016
Identification Method:	Blood collection tubes labeled with species, animal number, gender and collection date

3.2 Justification of Test System

Anticoagulated whole rabbit blood has historically been used for *in vitro* hemolysis testing.

4. Preparation of Standards and Controls

4.1 Dilution Factors for Calculations

Drabkin's Reagent was used as the hemoglobin reagent throughout the study. Throughout the course of the study, several dilutions of the whole blood or the blood plasma were conducted. To account for these in the calculations, the following dilution factors (DF) were used:

Plasma Hemoglobin Determination:

750 µL of plasma added to 750 µL of hemoglobin reagent

$$DF = \frac{\text{Final volume}}{\text{Volume plasma}} = \frac{1500\ \mu L\ \text{solution}}{750\ \mu L\ \text{plasma}} = 2$$

Whole Blood Hemoglobin Determination:

20 µL of whole blood added to 5 mL of hemoglobin reagent

$$DF = \frac{\text{Final volume}}{\text{Volume blood}} = \frac{5.02\ mL\ \text{solution}}{0.02\ mL\ \text{blood}} = 251$$

Diluted Blood Hemoglobin Determination:

300 µL diluted blood added to 4.5 mL of hemoglobin reagent

$$DF = \frac{\text{Final volume}}{\text{Volume diluted blood}} = \frac{4.8\ mL\ \text{solution}}{0.3\ mL\ \text{diluted blood}} = 16$$

Sample Hemoglobin Determination:

1.0 mL of Supernatant added to 1.0 mL of hemoglobin reagent

$$DF = \frac{\text{Final volume}}{\text{Volume supernatant}} = \frac{2.0\text{ mL solution}}{1.0\text{ mL supernatant}} = 2$$

Total Hemoglobin Concentration in each tube:

1.0 mL of diluted blood added to 7.0 mL CMF-PBS

$$DF = \frac{\text{Final volume}}{\text{Volume diluted blood}} = \frac{8.0\text{ mL}}{1.0\text{ mL}} = 8$$

4.2 Standards Preparation

The Human Hemoglobin Standard was dissolved in hemoglobin reagent. The reconstituted standard was tested at the following concentrations: 1.44, 0.800, 0.600, 0.300, 0.150, 0.0750, 0.0375, and 0.0188 mg/mL. The absorbance of the concentrations was read against a hemoglobin reagent blank in a spectrophotometer set at a wavelength of 540 nm. Using the information obtained from the absorbance readings and concentrations, a standard curve was generated.

4.3 Plasma Hemoglobin Determination

A 3.0 mL aliquot of the anticoagulated pooled blood was centrifuged at 700-800 × *g* for 15 minutes. A 750 µL portion of the plasma (supernatant) was added to 750 µL of hemoglobin reagent. The solution was allowed to stand for 15 minutes at room temperature and the absorbance was read at 540 nm. The plasma hemoglobin concentration of the blood sample was calculated from the prepared standard curve. The plasma hemoglobin concentration of the blood sample for this study was 0.2 mg/mL which met the requirements of the study.

4.4 Blood Hemoglobin Determination

Duplicate 20 µL portions of well-mixed, pooled whole blood with plasma hemoglobin <2.0 mg/mL were added to 5.0 mL aliquots of hemoglobin reagent. These solutions were allowed to stand for 15 minutes at room temperature and then the absorbance was read at 540 nm. The whole blood hemoglobin concentration was calculated from the standard curve.

The hemoglobin concentration of the pooled blood sample was adjusted to 10 ±1.0 mg/mL by diluting with an appropriate amount of CMF-PBS. The hemoglobin concentration was confirmed by taking 300 µL of the well-mixed, diluted blood and adding it to 4.5 mL of hemoglobin reagent in triplicate. The solutions were allowed to stand at room temperature for 15 minutes and the absorbance was read at 540 nm. The diluted blood hemoglobin concentration of the sample was calculated from the standard curve. The diluted blood hemoglobin concentration of the blood sample for this study was 10 mg/mL which met the requirements of the study.

5. Method

5.1 Test and Control Article Preparation

Only the plates were included in the preparation. Triplicate preparations of the test article and each of the controls were subjected to the preparation conditions as described in Tables 5 - 8. These were used for both the direct contact and extract portions of the test.

Table 5: Preparation of Test Article (Direct Contact and Extract)

Testing Portion	Vehicle	Ratio	Article Amount	Volume of Vehicle	Extraction Condition[1]
Extract	CMF-PBS	6 cm^2:1 mL (120 cm^2:20 mL)	67.6 cm^2	11 mL	121°C for 1 hour
Direct Contact	CMF-PBS	6 cm^2:1 mL (120 cm^2:20 mL)	67.6 cm^2	11 mL	NA

Table 6: Preparation of Negative Control (Direct Contact and Extract)

Testing Portion	Vehicle	Ratio	Article Amount	Volume of Vehicle	Extraction Condition[1]
Extract	CMF-PBS	3 cm^2:1 mL (60 cm^2:20 mL)	30.8 cm^2	10 mL	121°C for 1 hour
Direct Contact	CMF-PBS	3 cm^2:1 mL (60 cm^2:20 mL)	21 cm^2	7.0 mL	NA

Table 7: Preparation of Positive Control (Direct Contact and Extract)

Testing Portion	Vehicle	Volume of Vehicle	Extraction Condition[1]
Extract	SWFI	10 mL	121°C for 1 hour
Direct Contact	SWFI	7.0 mL	NA

Table 8: Preparation of Blank Control (Direct Contact and Extract)

Testing Portion	Vehicle	Volume of Vehicle	Extraction Condition[1]
Extract	CMF-PBS	10 mL	121°C for 1 hour
Direct Contact	CMF-PBS	7.0 mL	NA

[1]Extract portion only NA = Not Applicable

The following table contains a description of the test and control article extract conditions.

Table 9: Condition of Extracts

Time Observed	Extract	Condition of Extracts		
		Color	Clarity	Particulates
Before Extraction	Test	Colorless	Clear	No
	Negative Control	Colorless	Clear	No
	Positive Control	Colorless	Clear	No
	Blank	Colorless	Clear	No
After Extraction	Test	Colorless	Clear	No
	Negative Control	Colorless	Clear	No
	Positive Control	Colorless	Clear	No
	Blank	Colorless	Clear	No

The test article remained visually unchanged following the extraction process. The extracts were maintained at room temperature for less than 1 hour prior to use. The extracts were not centrifuged, filtered, or otherwise altered prior to dosing.

5.2 Test Procedure

Clot-free blood samples were collected from each animal into 7 mL vacuum tubes containing 12 mg of EDTA on the same day the test was performed. The blood collected from each animal was pooled into a borosilicate screw cap tube and mixed gently to prevent mechanical hemolysis.

The pooled blood was diluted with CMF-PBS to a total hemoglobin concentration of 10 ±1.0 mg/mL. Based on a ratio of 1.0 mL diluted blood to 7.0 mL vehicle, the following samples were prepared in triplicate:

Direct Contact

1.6 mL of diluted blood and 67.6 cm^2 of the test article in 11 mL CMF-PBS
1.0 mL of diluted blood and 21 cm^2 of the negative control in 7.0 mL CMF-PBS
1.0 mL of diluted blood and 7.0 mL of SWFI positive control
1.0 mL of diluted blood and 7.0 mL CMF-PBS (blank)

Extraction

1.0 mL of diluted blood and 7.0 mL of the CMF-PBS test article extract
1.0 mL of diluted blood and 7.0 mL of the CMF-PBS negative control extract
1.0 mL of diluted blood and 7.0 mL of SWFI positive control
1.0 mL of diluted blood and 7.0 mL of CMF-PBS (blank)

The samples were capped or covered, with the contents gently mixed by inversion or swirling, and then maintained for at least 3 hours at 37°C with periodic inversions at approximately 30 minute intervals. Following incubation, the direct contact blood-CMF-PBS mixtures were transferred to separate disposable centrifuge tubes. All tubes were centrifuged for 15 minutes at 700-800 × *g*. The condition of the supernatant was recorded and a 1.0 mL aliquot of each test article, negative control, positive control, and blank supernatant was added to individual 1.0 mL portions of Drabkin's reagent and allowed to stand for 15 minutes at room temperature. The condition of the supernatants was recorded a second time. The absorbance of each test article, negative control, positive control, and blank solution was measured at 540 nm using a spectrophotometer. The condition of the supernatants was then recorded a third, and final time.

The hemoglobin concentration of each test article, negative control and positive control solution was then calculated from the standard curve. The blank corrected percent hemolysis was calculated for each test article and the negative and positive controls as follows:

$$\text{Blank Corrected \% Hemolysis} = \frac{\text{ABS (Sample)} - \text{ABS (Blank)}}{\text{ABS (Diluted Blood)} - \text{ABS (Blank)}} \times 100$$

ABS = Absorbance

All times and temperatures reported herein are approximate and are within ranges established by the external standards described in the References section of this report and/or NAMSA standard operating procedures.

6. Evaluation and Statistical Analysis

The mean blank corrected % hemolysis (BCH) was calculated by averaging the blank corrected % hemolysis values of the triplicate test samples. In the event the BCH resulted in a value less than zero, the value was reported as 0.00. The standard deviation for the replicates was determined.

An average hemolytic index of the triplicate test samples was also calculated as follows:

Hemolytic Index = Mean BCH (Test Article) - Mean BCH (Negative Control)

A hemolytic grade was assigned based on the following scoring scheme:

Table 10: Test Scoring

Hemolytic Index	Hemolytic Grade
0 - <2%	Non-Hemolytic
2 - 5%	Slightly Hemolytic
>5%	Hemolytic

For the suitability of the system to be confirmed, the negative control must have had a mean blank corrected % hemolysis value <2% and the positive control must have had a mean blank corrected % hemolysis value of ≥5%. In the event the hemolytic index resulted in a value less than zero, the value was reported as 0.0.

7. Results

The values and observations obtained in this study are summarized below:

Table 11: Condition of Test, Control and Blank Supernatants

Test Article	Direct Contact			Extract		
	Color	Clarity	Particulates Present	Color	Clarity	Particulates Present
Prior to addition of Drabkin's	Pink	Clear	No	Colorless	Clear	No
Pre-spectrophotometric analysis	Light orange	Clear	No	Yellow	Clear	No
Post-spectrophotometric analysis	Light orange	Clear	No	Yellow	Clear	No

Positive Control	Direct Contact			Extract		
	Color	Clarity	Particulates Present	Color	Clarity	Particulates Present
Prior to addition of Drabkin's	Red	Clear	No	Red	Clear	No
Pre-spectrophotometric analysis	Red	Clear	No	Red	Clear	No
Post-spectrophotometric analysis	Red	Clear	No	Red	Clear	No

Negative Control	Direct Contact			Extract		
	Color	Clarity	Particulates Present	Color	Clarity	Particulates Present
Prior to addition of Drabkin's	Colorless	Clear	No	Colorless	Clear	No
Pre-spectrophotometric analysis	Yellow	Clear	No	Yellow	Clear	No
Post-spectrophotometric analysis	Yellow	Clear	No	Yellow	Clear	No

Blank Control	Direct Contact			Extract		
	Color	Clarity	Particulates Present	Color	Clarity	Particulates Present
Prior to addition of Drabkin's	Colorless	Clear	No	Colorless	Clear	No
Pre-spectrophotometric analysis	Yellow	Clear	No	Yellow	Clear	No
Post-spectrophotometric analysis	Yellow	Clear	No	Yellow	Clear	No

Table 12: Diluted Blood Sample Results

Sample	ABS 1	ABS 2	ABS 3
Diluted Blood	0.409	0.431	0.424

Table 13: Test and Control Direct Contact Sample Result

Sample	ABS 1	ABS 2	ABS 3	Mean Blank Corrected % Hemolysis	Standard Deviation	Mean Hemoglobin Concentration (mg/mL)	Hemolytic Index† (%)
Test Article	0.022	0.039	0.057	8.69	4.2	0.12	8.1
Negative Control	0.005	0.006	0.005	0.56	0.1	0.02	
Positive Control	0.454	0.456	0.456	108.13	0.3	1.38	
Blanks	0.003	0.003	0.003	0.71*	0.0		

Table 14: Test and Control Extract Sample Result

Sample	ABS 1	ABS 2	ABS 3	Mean Blank Corrected % Hemolysis	Standard Deviation	Mean Hemoglobin Concentration (mg/mL)	Hemolytic Index† (%)
Test Article	0.010	0.006	0.006	1.04	0.6	0.02	1.0
Negative Control	0.003	0.003	0.003	0.00	0.0	0.01	
Positive Control	0.414	0.410	0.408	97.45	0.7	1.24	
Blanks	0.003	0.003	0.003	0.71*	0.0		

= Not Applicable

* Mean % Hemolysis

† Hemolytic Index calculated as follows:

Test article mean blank corrected % hemolysis – Negative control mean blank corrected % hemolysis

8. Conclusion

The hemolytic index for the test article in direct contact with blood was 8.1%, and the hemolytic index for the test article extract was 1.0%. The test article in direct contact with blood was hemolytic and the test article extract was non-hemolytic.

Results and conclusions apply only to the test article tested. Any extrapolation of these data to other articles is the sponsor's responsibility.

9. Records

All raw data pertaining to this study and a copy of the final report are retained in designated NAMSA archive files in accordance with NAMSA SOPs.

10. References

American Society for Testing and Materials (ASTM) F756, Standard Practice for Assessment of Hemolytic Properties of Materials (2017).

International Organization for Standardization (ISO) 10993-4, Biological evaluation of medical devices - Part 4: Selection of tests for interactions with blood (2017).

International Organization for Standardization (ISO) 10993-12, Biological evaluation of medical devices - Part 12: Sample preparation and reference materials (2012).

International Organization for Standardization (ISO) 13485, Medical devices - Quality management systems - Requirements for regulatory purposes (2003/Technical Corrigendum 1 2009).

International Organization for Standardization/International Electrotechnical Commission (ISO/IEC) 17025, General requirements for the competence of testing and calibration laboratories (2005/Technical Corrigendum 1 2006).