Project:

Analysis of Platelet Aggregation by Cell Counting

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This SOP is based on the NCL Method ITA-2.1 - Analysis of Platelet Aggregation By Cell Counting, Marina A. Dobrovolskaia, Ph.D., Nanotechnology Characterization Laboratory, Frederick National Laboratory for Cancer Research, Leidos Biomedical Research, Inc., Frederick, MD 21702 USA – September 2015

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1 Introduction

This document describes a procedure for analysis of platelet aggregation. Platelets are small (~2 µm) anuclear cells obtained by fragmentation of megakaryocytes. Platelets, also known as thrombocytes, play a key role in hemostasis. Abnormal platelet counts and function may lead to either bleeding or thrombosis. Assessing nanoparticle effects on human platelets *in vitro* allows for quick screening of their potential anticoagulant or thrombogenic properties mediated by direct effects on platelets.

2 Principle of the Method

Platelet-rich plasma (PRP) is obtained from fresh human whole blood and incubated with either a control or test sample. Following incubation, PRP is analyzed using a cell or particle counter to determine the number of singular platelets. Percent aggregation is calculated by comparing the number of single (unaggregated) platelets in the test sample to the number of single (unaggregated) platelets in the control baseline sample (PPP).

3 Applicability and Limitations (Scope)

This assay is for the platelet activating (or activation inhibition) properties of nanoparticles. Care must be taken when analyzing nanoparticles that may interfere with the light transmission because of too high concentration.

4 Related Documents

Table 1:

Document ID	Document Title
EUNCL-ITA-2.2	Platelet aggregation by light transmission aggregometry

5 Equipment and Reagents

5.1 Equipment

5.1.1 Platelet aggregometer (PAP-4, MöLab)

5.1.2 Centrifuge capable of operating at 200 x g and 2,500 x g

5.1.3 Sysmex KX-21N Cellcounter

5.2 Reagents

5.2.1 Isoton diluent (Sysmex)

5.2.2 Freshly drawn human whole blood anticoagulated with sodium citrate

5.2.3 Collagen (OxProtect)

5.2.4 PBS, (GE Life Sciences, SH30256.01)

5.3 Materials

5.3.1 Pipettes covering range 0.5 to 1000 µL

5.3.2 silanised glass kuvettes (Mölab)

5.3.3 PPE tubes (3ml)

5.4 Preparation of Plasma, Test Samples and Controls

5.4.1. Test sample preparation

This assay requires 0.4 mL of nanoparticle solution, at 5X the highest test concentration. The nanoparticles should be dissolved/resuspended in PBS, which does not interfere with platelet aggregation.

The concentration is selected based on the plasma concentration of the nanoparticle at the intended therapeutic dose. For the purpose of this protocol this concentration is called "theoretical plasma concentration". Considerations for estimating theoretical plasma concentration were reviewed elsewhere and are summarized in Box 1 below.

Box 1. Example Calculation of Nanoparticle concentration for In Vitro Test

In this example, we assume the mouse dose is known to be 123 mg/kg.

$$human\ dose = \frac{mouse\ dose}{12.3} = \frac{123\frac{mg}{kg}}{12.3} = 10\ mg/kg$$

Blood volume constitutes approximately 8% of body weight, (e.g. a 70 kg human has approximately 5.6 L (8% of 70) of blood). This allows us to get a very rough estimation of what the maximum blood concentration may be.

$$in\ vitro\ concentration_{human\ maxtrix} = \frac{human\ dose}{human\ blood\ volume} = \frac{70\ kg\ \times 10\frac{mg}{kg}}{5.6\ L}$$

$$= \frac{700\ mg}{5.6\ L} = 0.125\ mg/mL$$

The assay will evaluate 4 concentrations: 10 X (or when feasible 100X, 30X or 5X) of the theoretical plasma concentration, theoretical plasma concentration and two 1:5 serial dilutions of

the theoretical plasma concentration. When the intended therapeutic concentration is unknown, the highest final concentration is 1 mg/mL or the highest reasonably achievable concentration.

For example if the final theoretical plasma concentration to be tested is 0.2 mg/mL, then a stock of 10 mg/mL will be prepared and diluted 10 fold (1 mg/mL), followed by two 1:5 serial dilutions (0.2 and 0.04 mg/mL). When 25 µL of each of these samples is added to the test tube and mixed with 0.1 mL of plasma, the final nanoparticle concentrations tested in the assay are: 2.0, 0.2, 0.04 and 0.008 mg/mL. Three 25 µL replicates are tested per each sample concentration.

5.4.2 Plasma preparation

You will need two types of plasma to perform this experiment: platelet rich plasma (PRP) and platelet poor plasma (PPP). Plasma from individual donors should be analyzed separately. Analysis of plasma from individual donors may be needed for mechanistic follow up experiments. Blood is drawn into monovette tubes containing sodium citrate as anticoagulant. Estimate the volume of PRP needed for this experiment based on the number of test samples. Keep in mind that each 10mL of whole blood produce ~2mL of PRP and ~5mL of PPP. First PRP and after that PPP is prepared by centrifugation Follow the guidance below for centrifugation time and speed used to prepare each type of plasma.

PRP – centrifuge whole blood at 200 x g for 8 minutes, collect plasma and transfer to a fresh tube; count the cells and prepare a stock of 300.000 platelets per µl by dilution with plasma (PPP)

PPP – centrifuge whole blood at 2,500 x g for 10 min, collect plasma and transfer to a fresh tube

Important: B) PRP must be prepared as soon as possible and no longer than 1 h after blood collection. PRP must be kept at room temperature and should be used within 2 hours. B) Exposure of either blood or PRP to cold temperature (< 20ºC) should be avoided, as it will induce platelet activation; likewise exposure to heat (> 37 °C) will activate platelets and affect the quality of test results.

5.4.3. Preparation of Controls

5.4.3.1. Negative Control (saline)

Sterile Ca^{2+}/Mg^{2+} free PBS is used as a negative control. Store at room temperature for up to 6 months.

5.4.3.2. Vehicle Control (relevant to the given nanoparticle)

When nanoparticles are formulated not in saline or PBS, the vehicle sample should be tested to estimate the effect of excipients on the platelet aggregation. This control is specific to each given nanoparticle sample. Vehicle control should match formulation buffer of the test nanomaterial by both the composition and

concentration. Dilute this sample the same way you dilute the test nanomaterials. If the vehicle is PBS this control can be skipped.

5.4.3.3. Positive Control

Several reagents listed below can be used as positive controls. The default positive control for this assay is collagen.

ADP – the stock concentration of adenosine diphosphate should be 5mM and stored at -20°C. Dilute ADP with PBS and use it in an final concentration of 5µM.
Collagen - Collagen is provided as a solution with a final concentration 200 µg/mL contained in an eppendorf tube. Keep it at maximal 4 °C. After opening the content of the vial should be used within 2 weeks. The temperature of unused collagen must be lower than 4°C!!!! Store it at -20°C.

Thrombin - the stock concentration of thrombin should be 100 U/ml and stored at -80°C. Dilute thrombin with PBS and use it in an final concentration of 1 U/ml.

6 Procedure

6.1 Prepare the platelet aggregometer instrument as described in the owner's manual.

6.1.1 Prepare PRP and PPP as described in step 5.4.2, then proceed to next step. The PRP count is adjusted to 300.000 platelets per µl per dilution with PPP (of the same donor!!!).

6.1.2 *Part A (Nanoparticle Ability to Induce Platelet Aggregation)*

In a silanised kuvette combine: 1) 200 µL PRP with 20 µL test material; 2) 200 µL PRP with 20 µL of positive control (collagen); 3) 200 µL PRP with 20 µL of negative control (PBS) and 4) 200 µL PRP with 20 µL of vehicle control (a buffer or media used to formulate nanoparticle; if nanoparticle is formulated in PBS, this sample can be skipped). Prepare three replicates for each sample.

Part B (Nanoparticle Ability to Interfere with Collagen-Induced Platelet Aggregation)

In a separate set of tubes combine: 1) 200 µL of PRP with 20 µL of negative control (PBS); 2) 200 µL of PRP with 10 µL of positive control (collagen) plus10 ul of PBS; 3)

200 μL of PRP with 10 μL of positive control (collagen) and 10 μL of test nanomaterial; 4) 200 μL PRP with 20 μL of vehicle control (a buffer or media used to formulate nanoparticle; if nanoparticle is formulated in PBS, this sample can be skipped). Prepare three replicates for each test combination. \

Note: the final concentration of nanoparticles in this case is lower (half!!!) than that tested in part A. If there is a reason to expect that this difference will affect the test results, adjust the concentration of the stock nanoparticles accordingly.

Part C (Assessment of Nanoparticle Interference with the assay)

Prepare 1 control tube, by combining 200 μL of platelet poor plasma (PPP) and 20 μL of nanoparticle solution. *If nanoparticles aggregate to a micron size particulates, they either create artificially high number of single platelet count (if the aggregates resemble platelet size and pass the aperture) or will not pass through the aperture and prevent accurate counting of single platelets, resulting in false-negative or false-positive result, respectively.*

6.1.3 The cuvettes, including a special magnet stirrer and 200µl of adjusted PRP are placed in the aggregometer and prewarmed for 60 seconds at 37°C. Parallel the baseline is set with PPP. After that the cuvettes are placed in the measurement chamber the test material / the negative control / the positive control is admitted and incubated with stirring for 5 minutes. After that the samples are analyzed immediately in the cell counter.

6.2 Flow chart

1. Prepare/prewarm aggregometer	Warm up the aggregometer 15 minutes before use
2. Blood preparation	Take blood from at least 3 healthy donors and prepare PRP and PPP by centrifugation
3. Prepare experimental controls	Positive control: PPP Negative control: PRP; PBS Vehicle control: nanomaterial in the maximal concentration in PPP
4. Prepare the platelet solution	Adjust PRP to 300.000 platelets/µl with autologues PPP
5. Experimental procedure	- add 20µl each of 4 different concentration of test material to 200µl PRP each - add 20µl positive control to 200µl PRP - add 20µl negative control to 200µl PRP - add 20µl vehicle control to 200µl PRP
6. Aggregation	Place cuvettes in the aggregometer and „incubate“ them for at least 5 minutes
7. Cell counting	The cells in the supernatant in the cuvettes are counted in the cell counter

6.3 Data Analysis and Calculations

The following parameters should be calculated for each control and test sample:

6.3.1 Percent Coefficient of Variation:

$$\frac{SD}{Mean} \times 100\%$$

6.3.2 Percent Platelet Aggregation:

$$\frac{(\text{Platelet Count}_{\text{negative control}} - \text{Platelet Count}_{\text{test sample}})}{\text{Platelet Count}_{\text{negative control}}} \times 100\%$$

6.3.4 Positive sample - Assay threshold is 20%. Percent platelet aggregation values above 20% are considered positive, i.e. test material induces platelet aggregation.

6.3.5 Inhibition of collagen-induced platelet aggregation – there is no formal guidance on what degree of inhibition is considered significant. Apply scientific judgement to interpret results from the part B of the study. Statistically significant inhibition does not necessarily mean it is physiologically relevant. If an inhibition is observed one should consider relevant follow up *in vivo* study to verify *in vitro* findings.

7 Quality Control, Quality Assurance, Acceptance Criteria

7.1 %CV for each control and test sample should be within 25%.

7.2 If both replicates of positive control or negative control fail to meet acceptance criterion described in 7.1, the run should be repeated.

7.3 Within the acceptable run, if two of three replicates of unknown sample fail to meet acceptance criterion described in 7.1, this unknown sample should be re-analyzed.

8 Health and Safety Warnings, Cautions and Waste Treatment

8.1 Universal precautions must be used when handling human peripheral blood. No sharps are to be used in the preparation of cell isolates of plasma from human healthy volunteer blood. All liquid waste must be discarded into 1% Virkon solution and left overnight (18 hours) in a class II BSC with the lid loose to allow for venting.

9 Abbreviations

CV	coefficient of variation
PBS	phosphate buffered saline
PRP	platelet rich plasma
PPP	platelet poor plasma
SD	standard deviation

10 References