NCL Method ITA-12

In vitro Analysis of Nanoparticle Effects on Plasma Coagulation Time

Nanotechnology Characterization Laboratory
Frederick National Laboratory for Cancer Research
Leidos Biomedical Research, Inc.
Frederick, MD 21702
(301) 846-6939
ncl@mail.nih.gov
http://www.ncl.cancer.gov

https://ncl.cancer.gov

This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

Method written by:
Edward Cedrone[1],
Timothy M. Potter[1],
Barry W. Neun[1]
Marina A. Dobrovolskaia[1,*]

1 - Nanotechology Characterization Lab., Frederick National Laboratory for Cancer Research, Frederick, MD 21702

*- address corespondence to: marina@mail.nih.gov

1. Introduction

This document describes a protocol for assessing the effect a nanoparticle formulation may have on plasma coagulation time. Coagulation, i.e., blood clotting, is a highly complex process that involves many components. There are three main pathways for coagulation: intrinsic (also known as the contact activation pathway, because it is activated by a damaged surface); extrinsic (also known as the tissue factor pathway); and the final common pathway. Each pathway can be assessed by a specialized test. For example, the activated partial thromboplastin time (APTT) assay is used to assess the intrinsic pathway, while the prothrombin time (PT) assay is a measure of the extrinsic pathway. Extrinsic and intrinsic pathways converge into common pathway. Thrombin time (TT) is an indicator of the functionality of the final common pathway. Each pathway involves many coagulation factors, some of which overlap between pathways. The APTT assay assesses functionality of factors XII, XI, IX, VIII, X, V, II. The PT assay assesses activity of factors VII, X, V and II. All three assays assess the role of fibrinogen.

2. Principles

This assay describes the analysis of plasma coagulation via three separate tests: prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin time (TT). Nanoparticles are incubated with fresh human plasma and assayed for coagulation time, compared to standard controls for each assay, using a coagulometer. When normal plasma is exposed to nanomaterials in vitro, and it results in depletion or inhibition of a certain coagulation factor, a delay in plasma coagulation is expected.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol and their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted. Please note that suppliers may undergo a name change due to a variety of factors. Brands and part numbers typically remain consistent but may also change over time.

3.1 Reagents

3.1.1 Human blood from at least 3 donors, anti-coagulated with sodium citrate

3.1.2 Neoplastine Cl (Diagnostica Stago, 00667)

3.1.3 Thrombin (Diagnostica Stago, 00611)

3.1.4 $CaCl_2$ (0.025M) (Diagnostica Stago, 00367)

3.1.5 Owren-Koller Buffer (Diagnostica Stago, 00360)

3.1.6 PTT-A (Diagnostica Stago, 00595)

3.1.7 CoagControl N+ABN (Diagnostica Stago, 00677)

3.1.8 RPMI (GE Life Sciences, HyClone, SH30096.01)

3.1.9 PBS (GE Life Sciences, Hyclone, SH 30256.01)

3.2 Materials

3.2.1 Metal balls for coagulometer (Diagnostica Stago, 26441)

3.2.2 Pipettes covering the range of 0.05 to 10 mL

3.2.3. Finntip, 1.25 mL (Fisher, NC0298434)

3.2.4. 4-well cuvettes (Diagnostica Stago, 38876)

3.3 Equipment

3.3.1 Centrifuge capable of operating at 2,500 x g

3.3.2 Refrigerator, 2-8ºC

3.3.3 Diagnostica Stago Art4 Coagulometer

4. Preparation of Study Samples

This assay requires 500 μL of nanoparticles, at a concentration 10x that of the highest tested concentration, dissolved/resuspended in PBS or other relevant media. 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 (1) 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 20 mg/mL will be prepared and diluted 10-fold (2 mg/mL), followed by two 1:5 serial dilutions (0.4 and 0.08 mg/mL). When 0.1 mL of each of these samples is added to the test tube and mixed with 0.9 mL of plasma, the final nanoparticle concentrations tested in the assay are: 2.0, 0.2, 0.04 and 0.008 mg/mL.

5. Preparation of Test, Normal and Abnormal Control Plasmas

5.1 Test-Plasma

Use freshly collected whole blood within 1 h after collection. Spin the blood 10 min, 2500 x g at 20-22ºC; collect plasma and pool from at least 2 donors. Pooled plasma is stable for 8 h at room temperature. Do not refrigerate or freeze. The assay can also be performed in the plasma from individual donors when needed for mechanistic follow up experiments.

Analyze 2 duplicates (4 total samples) of test-plasma in each of the coagulation assays; run one duplicate before the nanoparticle samples and the second duplicate at the end of each run to verify that the plasma functionality is not affected throughout the duration of the experiment.

5.2 Nanoparticle-treated test-plasma

In a microcentrifuge tube, combine 100 μL of nanoparticles (as prepared in step 4) and 900 μL of test plasma; mix well and incubate 30 minutes at 37ºC. Prepare three tubes for each test sample (i.e. when each nanoparticle is tested at 4 concentrations, one needs 3 tubes for each concentration, a total 12 tubes per test-nanoparticle).

Note: Insoluble nanoparticles, which can be separated from the bulk plasma by centrifugation may be removed by spinning the test tubes for 5 min at 18,000 x g. it is assumed that any proteins involved in the coagulation process and adsorbed onto the particle surface will be removed from the sample in this step and the consequences of such binding on the plasma coagulation pathways will be assessed. Often nanoparticles are soluble or modified with poly(ethylene glycol), and therefore cannot be easily separated from plasma at the end of the incubation step. In this case, the sample analysis proceeds to the next step without centrifugation.

5.3 Normal and Abnormal (Coag N+ABN) control plasmas

Reconstitute lyophilized control plasmas with 2 mL of distilled water. Let the solutions stand at room temperature 30 min prior to use. Mix thoroughly before use. Keep unused portion refrigerated and use within 48 h after reconstitution. These plasma samples are used as instrument controls.

5.4. Reagents used to initiate plasma coagulation (Neoplastin, PTT-A Reagent, Thrombin)

These reagents are supplied as lyophilized powder. Reconstitute according to the manufacturer's instructions and use fresh or refrigerate and use within the time specified by the manufacturer.

6. Experimental Procedure

6.1 Set-up instrument test parameters for each of the four assays. Refer to the Appendix for a quick list of instrument settings and reagent volumes. Allow the instrument to warm up for 5-10 min prior to use.

6.2 Prepare all reagents and warm to 37ºC prior to use. Note that lyophilized reagents should be reconstituted at least 30 minutes prior to use.

6.3 Place cuvettes into A, B, C and D test rows on the coagulometer *(Note: this protocol is based on the semi-automatic STArt4 coagulometer from Diagnostica Stago* (2). *If using a different instrument, please follow the operational guidelines recommended by the instrument manufacturer)*.

6.4 Add one metal ball into each cuvette and allow cuvette and ball to warm for at least 3 minutes prior to use.

6.5 Add 100 µL of control or test plasma to a cuvette when testing PT or thrombin time, and 50 µL when testing APTT (refer to the Appendix for reference). Prepare 2 wells for each test-tube prepared in step 5.2

6.6 <u>This step is only for APTT:</u>

Add 50 µL of PTT-A reagent to plasma samples in cuvettes.

6.7 Start the timer for each of the test rows by pressing the A, B, C or D timer buttons. Ten seconds before time is up, the timer starts beeping. When this happens, immediately transfer cuvettes to PIP row and press PIP button to activate pipettor.

6.8 When time is up, add coagulation activation reagent to each cuvette and record coagulation time. Refer to the Appendix for the type of coagulation activation reagent and volume for each of the four assays.

7. Calculations and data interpretation

7.1 A Percent Coefficient of Variation should be calculated for each control or test according to the following formula: %CV = SD/Mean x 100%

7.2. Normal and Abnormal control plasma should coagulate within the time established by the certifying laboratory (e.g. for the most batches of control plasmas normal coagulation time in the PT assay is ≤ 13.4 seconds, APTT – ≤ 34.1 second and Thrombin – ≤ 21 seconds; abnormal control plasma coagulation time should be above these limits). When normal and abnormal control perform as described above and untreated plasma sample coagulates within normal time limits, both the instrument and the test plasma are qualified for the use in this test

7.3. Nanoparticles have no effect on the assay coagulation cascade when coagulation time of the test plasma samples after exposure to nanoparticles is within the normal limits.

7.4. Prolongation of the plasma coagulation time in plasma samples exposed to nanoparticles suggests that test-particle either deplete or inhibit coagulation factors. There is no guidance on the degree of prolongation, but in general prolongation of 2-fold or more than that in untreated control is considered physiologically significant

8. Acceptance Criteria

8.1 %CV for each control and test sample should be within 5%.

8.2 If two duplicates of the same study sample demonstrated results > 5% different, this sample should be reanalyzed.

9. References

1. Dobrovolskaia MA, McNeil SE. Understanding the correlation between in vitro and in vivo immunotoxicity tests for nanomedicines. J Control Release. 2013 Dec 10;172(2):456-66
2. STart4 Standard operating procedure and training manual. Diagnostica Stago, 26987, June 2002.

10. Abbreviations

ABN	abnormal
API	active pharmaceutical ingredient
APPT	activated partial thromboplastin time
CV	coefficient of variation
NCL	Nanotechnology Characterization Laboratory
P	pathologic
N	normal
PT	prothrombin time
SD	standard deviation
TT	thrombin time

11. Appendix

ITA-12 Quick Reference Guide

Assay	Control	Instrument Settings				Volumes		Normal Coagulation Time
		Max Time	Incubation Time	Single/ Duplicate	Precision	Plasma and Reagent Volumes	Coagulation Activation Reagent Volumes	
PT (neoplastine)	Coag Control N+ABN	60 sec	120 sec	Duplicate	5%	100 µL Plasma	Neoplastine Reagent: 100 µL (PIP Position 4)	≤ 13.4 sec
APTT	Coag Control N+ABN	120 sec	180 sec	Duplicate	5%	50 µL Plasma + 50 µL PTTA Reagent	$CaCl_2$: 50 µL (PIP Position 2)	≤ 34.1 sec
Thrombin	Coag Control N+ABN	60 sec	60 sec	Duplicate	5%	100 µL Plasma	Thrombin: 100 µL (PIP Position 4)	≤ 21 sec