



NCL Method ITA-12

Analysis of Nanoparticle Effects on Plasma Coagulation Times in Vitro

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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.

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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 the 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, and 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, assayed for coagulation time using a coagulometer, and compared to standard controls for each assay. When normal plasma is exposed to nanomaterials in vitro which deplete or inhibit certain coagulation factors, a delay in plasma coagulation is expected.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted.

3.1 Reagents

1. Human blood from at least three donors, anti-coagulated with sodium citrate
2. Neoplastine Cl (Diagnostica Stago, 00666)
3. Thrombin (Diagnostica Stago, 00611)

4. CaCl₂ (0.025 M) (Diagnostica Stago, 00367)
5. Owren-Koller Buffer (Diagnostica Stago, 00360)
6. PTTA (Diagnostica Stago, 00595)
7. CoagControl N+ABN (Diagnostica Stago, 00676)
8. RPMI-1640 (Invitrogen, 11835-055)
9. PBS (GE Life Sciences, SH 30256.01)

3.2 Materials

1. Metal balls for coagulometer (Diagnostica Stago, 26441)
2. Pipettes, 0.05 to 10 mL
3. Finntip, 5 mL (ThermoScientific, 9404180)
4. 4-well cuvettes (Diagnostica Stago, 38876)

3.3 Equipment

1. Centrifuge, 2,500xg
2. Refrigerator, 2-8°C
3. DiagnosticaStago Art4 Coagulometer

4. Preparation of Study Samples

This assay requires 0.5 mL of nanoparticle solution dissolved/resuspended in complete culture medium, at a concentration 10X the highest final test concentration. Test concentrations are based on the calculated 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 have been reviewed elsewhere [1] and are summarized in Box 1 below.

This assay evaluates four concentrations: 10X (5X if 10X cannot be achieved, or 100X or 30X when feasible) of the theoretical plasma concentration, the theoretical plasma concentration, and two serial 1:5 dilutions of the theoretical plasma concentration. When the intended therapeutic concentration is unknown, 1 mg/mL is used as the highest concentration. Alternatively, the highest reasonably achieved concentration can be used if 1 mg/mL is unattainable.

For example, if the theoretical plasma concentration to be tested is 0.2 mg/mL, a stock of 20 mg/mL is prepared. This sample is then 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 sample dilutions is added to the test tube and mixed with 0.9 mL of plasma, the final nanoparticle concentrations tested in this assay are: 2.0, 0.2, 0.04 and 0.008 mg/mL.

Box 1. Example Calculation to Determine Nanoparticle Theoretical Plasma Concentration

In this example, we are assuming a known efficacious mouse dose of 123 mg/kg. Therefore, the scaled equivalent human dose would be:

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

The blood volume of a human is approximately 8% of the body weight. Therefore, an average human of 70 kg body weight has approximately 5.6 L of blood. Assuming all the nanoparticle injected goes into the systemic circulation, this provides a rough approximation of the potential maximum nanoparticle concentration in a human. The theoretical plasma concentration, i.e. in vitro test concentration, is calculated by:

$$\text{theoretical plasma concentration} = \frac{\text{human dose}}{\text{human blood volume}} = \frac{70 \text{ kg} \times 10 \text{ mg/kg}}{5.6 \text{ L}} = 0.125 \text{ mg/mL}$$

5. Preparation of Test, Normal and Abnormal Control Plasmas

5.1 Test-Plasma

Use freshly collected whole blood within 1 hr after collection. Spin the blood 10 min, 2500xg at 20-22°C; collect plasma and pool from at least two donors. Pooled plasma is stable for 8 hr at room temperature. Do not refrigerate or freeze. The assay can also be performed in plasma from individual donors when needed for mechanistic follow up experiments. Analyze two duplicates (four 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 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 37°C. Prepare three tubes for each test sample (i.e., when each nanoparticle is tested at four concentrations, three

tubes for each concentration are needed, for a total of twelve tubes per test-nanoparticle).

Note: Insoluble nanoparticles can be separated from the bulk plasma by centrifuging the test tubes for 5 min at 18,000xg. 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. Allow the solutions to stand at room temperature 30 min prior to use. Mix thoroughly before use. Keep unused portion refrigerated and use within 48 hr after reconstitution. These plasma samples are used as instrument controls.

5.4 Neoplastin, PTTa-Reagent, and Thrombin

These are reagents to initiate plasma coagulation. They 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

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 5-10 min prior to use.
2. Prepare all reagents and warm to 37°C prior to use. Note that lyophilized reagents should be reconstituted at least 30 minutes prior to use.
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*).

4. Add one metal ball into each cuvette and allow cuvette and ball to warm for at least 3 minutes prior to use.
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. This step is only for APTT : Add 50 μL of PTTa reagent to plasma samples in cuvettes.
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.
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

1. A Percent Coefficient of Variation should be calculated for each control or test according to the following formula:

$$\%CV = SD/\text{Mean} \times 100\%$$

2. Normal and Abnormal control plasma should coagulate within the time established by the certifying laboratory. For most batches of control plasmas, normal coagulation time in the PT assay is ≤ 13.4 sec, APTT ≤ 34.1 sec, and Thrombin ≤ 21 sec. Abnormal control plasma coagulation times should be above these limits. When normal and abnormal controls perform as described above and untreated plasma sample coagulates within the normal time limits, both the instrument and the test plasma are qualified for the use in the assay.
3. Nanoparticles have no effect on the assay coagulation cascade when coagulation times of the test plasma samples are within the normal limits after exposure to nanoparticles.
4. Prolongation of plasma coagulation times in plasma samples exposed to nanoparticles suggests the test particle either depletes or inhibits coagulation factors. There is no guidance on the degree of prolongation, but generally prolongation ≥ 2 -fold versus untreated control is considered physiologically significant.

8. Acceptance Criteria

1. The %CV for each control and test sample should be within 5%.
2. If two duplicates of the same study sample demonstrate 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;172(2):456-66.
2. SStart4 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
Thrombine	Coag Control N+ABN	60 sec	60 sec	Duplicate	5%	100 µL Plasma	Thrombine: 100 µL (PIP Position 4)	≤ 21 sec