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, 2-8ºC
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.

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 thrombine 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:
         \[
         \%CV = \frac{SD}{\text{Mean}} \times 100\%
         \]
   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 \( \leq 13.4 \) sec, APTT \( \leq 34.1 \) sec, and Thrombin \( \leq 21 \) 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 \( \geq 2 \)-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


10. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ABN</td>
<td>abnormal</td>
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<tr>
<td>API</td>
<td>active pharmaceutical ingredient</td>
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<tr>
<td>APPT</td>
<td>activated partial thromboplastin time</td>
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<td>CV</td>
<td>coefficient of variation</td>
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<td>NCL</td>
<td>Nanotechnology Characterization Laboratory</td>
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<tr>
<td>P</td>
<td>pathologic</td>
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<tr>
<td>N</td>
<td>normal</td>
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<tr>
<td>PT</td>
<td>prothrombin time</td>
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<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>TT</td>
<td>thrombin time</td>
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11. Appendix

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<table>
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<tr>
<th>Assay</th>
<th>Control</th>
<th>Instrument Settings</th>
<th>Volumes</th>
<th>Normal Coagulation Time</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Max Time</td>
<td>Incubation Time</td>
<td>Single/Duplicate</td>
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<tr>
<td>PT (neoplastine)</td>
<td>Coag Control N+ABN</td>
<td>60 sec</td>
<td>120 sec</td>
<td>Duplicate</td>
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<tr>
<td>APTT</td>
<td>Coag Control N+ABN</td>
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<td>180 sec</td>
<td>Duplicate</td>
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<tr>
<td>Thrombine</td>
<td>Coag Control N+ABN</td>
<td>60 sec</td>
<td>60 sec</td>
<td>Duplicate</td>
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