

Project:



Leukocyte Procoagulant Activity

In vitro Induction of Leukocyte Procoagulant Activity by Nanoparticles

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1 Introduction

Leukocyte procoagulant activity (PCA) is accepted as an important component in the onset of disseminated intravascular coagulation (DIC). DIC is common in acute promyelocytic leukemia (APL) and other forms of cancer [1-5]. DIC in cancer patients is often observed after initiation of therapy with cytotoxic oncology drugs that act by altering DNA replication (e.g., doxorubicin, daunorubicin, and vincristin) [3, 6]. Cytotoxic oncology drugs acting by other mechanisms, (e.g., methotrexate and paclitaxel) do not induce DIC [7-8]. DIC is also a common complication in sepsis [9-12]. Cytotoxic drugs (doxorubicin, vincristin, and daunorubicin) and endotoxin have previously been shown to induce leukocyte PCA *in vitro* and DIC *in vivo* [13-21]. *In vitro*, doxorubicin-induced leukocyte PCA has previously been linked to DIC *in vivo* [3].

2 Principle of the Method

This document describes a protocol for assessing the ability of a nanoparticle formulation to induce leukocyte procoagulant activity. This protocol includes two cell models;

- normal leukocytes, represented by PBMC isolated from blood of healthy donor volunteers.
- acute promyelocytic leukemia cells, represented by the HL-60 cell line.

Briefly, cells are treated with nanoparticles, and then undergo a wash step to remove excess particles. Isolated cells are then used to initiate plasma coagulation, which is measured using coagulometer (following EU-NCL protocol EUNCL-ITA-012) for analysis of prothrombin time.

3 Applicability and Limitations (Scope)

This methodology is for the *in vitro* assessment of nanoparticle induction of Leukocyte PCA. The mechanism of any induction must be determined in follow up experiments. Endotoxin present in nanoparticle preparations may induce non-particle specific PCA so care must be taken in nanoparticle preparation. Cells must be kept at room temperature prior to warming to 37°C for experimentation. Keeping cells at 37°C for longer period of time may result in cells sticking to universal tubes, subsequent lower cell densities in experiments and therefore no induction of PCA.

4 Related Documents

Table 1:

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5 Equipment and Reagents

5.1 Equipment

- 5.1.1 Centrifuge
- 5.1.2 Refrigerator, 2-8°C
- 5.1.3 Freezer, -20°C
- 5.1.4 Cell culture incubator with 5% CO₂ and 95% humidity.
- 5.1.5 Biohazard safety cabinet approved for level II handling of biological material
- 5.1.6 Inverted microscope
- 5.1.7 Vortex
- 5.1.8 Equipment for cell counting
- 5.1.9 Merlin MC4+ coagulometer or comparable

5.2 Materials

- 5.2.1 Pipettes covering the range of 0.05 to 10 mL
- 5.2.2 6-well plates
- 5.2.3 Polypropylene tubes, 50 and 15 mL
- 5.2.4. Finntip, 5mL (ThermoScientific, 9404180)
- 5.2.5. 4-well cuvettes (Diagnostica Stago, 38876)
- 5.2.6 Polystyrene tubes, 5mL (Falcon, 352058)
- 5.2.7 Scintillation vials, 20mL (Research Products International, FS74511-20)

5.3 Reagents

- 5.3.1 Human blood from at least 3 donors, anti-coagulated with Li-heparin for PBMC isolation and anti-coagulated with Na-citrate for plasma coagulation test
- 5.3.2 Ficoll-Paque Plus (GE Life Sciences, 17-1440-02)
- 5.3.3 Phosphate buffered saline (PBS) (GE Life Sciences, SH30256.01)

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- 5.3.4 Ultrapure LPS (InvivoGen, tlr-pektps)
- 5.3.5 Doxorubicin hydrochloride (Bedford Labs, NIH DVR pharmacy)
- 5.3.6 Fetal bovine serum (FBS) (GE Life Sciences, Hyclone , SH30070.03)
- 5.3.7 RPMI-1640 (Invitrogen, 11835-055)
- 5.3.8 Hanks balanced salt solution (HBSS) (Invitrogen, 24020-117)
- 5.3.9 Pen/Strep solution (Invitrogen, 15140-148)
- 5.3.10 β -mercaptoethanol (Sigma, M7522)
- 5.3.11 L-glutamine (Hyclone, SH30034.01)
- 5.3.12 Trypan Blue solution (Invitrogen, 15250-061)
- 5.3.13 Neoplastine Cl (Diagnostica Stago, 00666)
- 5.3.14 CoagControl N+ABN (Diagnostica Stago, 00676)
- 5.3.15. Calcium ionophore (Sigma, C7522)

5.4 Cell lines

- 5.4.1 HL-60 promyelocytic cells (ATCC, CCL-240)

5.5 Reagent Preparation

5.5.1 Complete RPMI-1640 medium

The complete RPMI medium should contain the following reagents:

- FBS (heat inactivated) 10% for PBMC and 20% for HL-60
- 2 mM L-glutamine
- 100 U/mL penicillin
- 100 μ g/mL streptomycin sulfate

Store at 2-8°C protected from light for no longer than 1 month.

Before use, warm in a water bath.

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5.5.2 Lipopolysaccharide, 1 mg/mL (LPS, Stock)

Add 1 mL of sterile PBS or cell culture medium per 1 mg of LPS to the vial and vortex to mix.

Store daily use aliquots at a nominal temperature of -20°C.

5.5.3 Doxorubicin, stock

Doxorubicin (adriamycin) is provided as a solution at 2 mg/mL and stored at 2-8°C.

5.5.4 Positive Controls for cell culture

Positive control for PBMC

Dilute stock LPS solution in cell culture medium to a final concentration of 1 µg/mL.

- Store at room temperature.
- Discard unused portion after experiment.
- Alternatively, calcium ionophore at the final concentration of 50 ng/mL can be used.

Positive control for HL-60 cells

Dilute doxorubicin stock solution in cell culture medium to a final concentration of 50 µg/mL.

- Store at room temperature.
- Discard unused portion after experiment.
- Alternatively, calcium ionophore at the final concentration of 50 ng/mL can be used.

5.5.5 Negative Control

Use PBS as a negative control. Process it the same way as your study samples.

5.5.6 Heat-inactivated fetal bovine serum

- Thaw a bottle of FBS at room temperature, or
- overnight at 2-8°C and allow to equilibrate to room temperature.

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- Incubate 30 minutes at 56°C in a water bath, mixing every 5 minutes.
- Fifty (50) mL single use aliquots may be stored at 2-8°C for up to one month or at a nominal temperature of -20°C indefinitely.

5.5.7 Buffer A

Prepare Buffer A by dissolving NaCl to a final concentration of 150 mM and CaCl₂ to a final concentration of 6.6 mM in 20 mM HEPES, pH 7.4.

5.5.8 Preparation of coagulation controls (Coag N+ABN):

Reconstitute lyophilized control plasmas with 1 mL of distilled water.

- Let the solutions stand at room temperature 30 minutes 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.

5.5.9 Preparation of plasma for coagulation test:

Use freshly collected whole blood anti coagulated with Na-citrate (blue top vacutainers) within 1 hour after collection.

- Spin the blood 10 min at 2500 x g at 20-22°C; collect plasma and pool.
- Pooled plasma is stable for 8 h at RT. Do not refrigerate or freeze.

Analyze 2 duplicates of test-plasma in each coagulation assay,

- run one duplicate before nanoparticle-treated plasma samples and
- the second duplicate at the end of each run.

5.5.10 Vehicle Control

Vehicle control is the buffer or media used to formulate test nanomaterials. Common excipients used in nanoformulations are trehalose, sucrose, and albumin. However, other reagents and materials are also used alone or in combination. Vehicle control should match formulation buffer of the test-nanomaterial by both composition and concentration. This control can be skipped if nanoparticles are stored in PBS.

5.5.11 Neoplastin reagent

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This reagent is supplied as lyophilized powder along with reconstitution buffer. Reconstitute according to the manufacturer’s instructions and use fresh or refrigerate and use within the time specified by the manufacturer.

5.6 Preparation of Study Samples

This assay requires 3 mL of nanoparticles or 1.5mL if using only PBMC or HL-60 model. The particles should be dissolved/resuspended in complete culture medium at a concentration 10 X of the highest tested concentration. 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 (22) 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.

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

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

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 400 µL of each of these samples are combined in a culture plate well with 3.6mL

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of cells, the final concentrations of nanoparticles are 0.008, 0.04, 0.2, and 2mg/mL. Each nanoparticle concentration is plated 2 times.

6 Procedure

6.1 Cell Preparation – Isolation of Human Lymphocytes

6.1.1 Place freshly drawn blood anticoagulated with Li-heparin (green top vacutainers) into 15 or 50 mL conical centrifuge tubes; add an equal volume of room-temperature PBS and mix well.

6.1.2 Slowly layer the Ficoll-Paque solution underneath the blood/PBS mixture by placing the tip of the pipet containing the Ficoll-Paque at the bottom of the blood sample tube. Alternatively, blood/PBS mixture may be slowly layered over Ficoll-Paque solution. Use 3 mL of Ficoll-Paque solution per 4 mL of blood/PBS mixture.

Note: To maintain Ficoll-blood interface it is helpful to hold tube at 45° angle.

6.1.3 Centrifuge 30 minutes at 900 x g, 18-20°C without brake.

6.1.4 Using a sterile pipet, remove the upper layer containing plasma and platelets, and discard.

6.1.5 Using a fresh sterile pipet, transfer mononuclear cell layer into another centrifuge tube.

6.1.6 Wash cells by adding an excess of HBSS; centrifuge for 10 min at 400 x g, 18-20°C. The HBSS volume should be ~3 times the volume of the mononuclear layer.

Note: Typically 4 mL of blood/PBS mixture results in ~2 mL of mononuclear layer and requires 6 mL of HBSS for the wash step.

6.1.7 Discard supernatant and repeat wash step once more.

6.1.8 Resuspend cells in complete RPMI 1640 medium. Count an aliquot of cells and determine viability using trypan blue exclusion.

6.1.9 If cell viability is $\geq 80\%$, dilute cells in complete culture media to the concentration of 3×10^6 cells/mL and proceed to step 7.1.1.

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6.2 Cell Preparation - HL-60 Cells

- 6.2.1 Grow cells in complete culture media; avoid a cell density greater than 1×10^6 cells/mL. On the day of experiment, count cells; if cell viability is $\geq 80\%$, concentrate cells in complete culture media to the concentration of 3×10^6 cells/mL and proceed to step 7.1.1.

6.3 Experimental Procedure - Cell treatment with nanoparticles and controls

- 6.3.1 Aliquot 3.6 mL of cell suspension into each well of a 6 well plate.
- 6.3.2 Add 400 μ L of test nanoparticle, positive control and negative control to respective wells. Negative control for both cell models is PBS. Positive control for PBMC is LPS at final concentration of 1 μ g/mL. Positive control for HL-60 cells is doxorubicin at a final concentration of 50 μ g/mL. Ca^{2+} ionophore can also be used as a positive control. Prepare two wells for each sample. See Appendix for example plate map.
- 6.3.3 Incubate cells with nanoparticles and controls. Time of incubation is 5 hours for the HL-60 model and 24 h for PBMC.
- 6.3.4 At the end of the incubation time, remove cells from the incubator, transfer cells into 5mL falcon tubes, and wash cells two times with 1 mL of PBS. For each wash cycle spin cells at 400 x g for 5 min
- 6.3.5 After last wash, reconstitute cell pellet in 1mL of Buffer A which usually results in cell concentration of 10×10^6 cells/mL and transfer cells into 20mL scintillation vial or equivalent

Note: any vial or tube can be used as long as it fits into coagulometer warming well

- 6.3.6 Keep cell suspensions at room temperature; place in the incubator (37°C) 5-10 minutes prior to testing to warm up. The sample will then be transferred to the 37°C chamber on the coagulometer when ready to start.

6.4 Experimental Procedure - Determining plasma coagulation time

- 6.4.1 Set-up the instrument test parameters as shown below:

Max Time: 500 sec

Incubation Time: 120 sec

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Single/Duplicate: Duplicate

Precision: 5%

Allow instrument to warm up to 5-10 minutes prior to use.

- 6.4.2 Prepare all reagents and cells, and warm them up to 37°C prior to use. Note that lyophilized reagents should be reconstituted at least 30 minutes prior to use. It is not advised to keep more than 10 cell samples at 37°C at one time.
- 6.4.3 Place cuvettes into A, B, C and D test rows on coagulometer (Note this protocol is based on semi-automatic STart4 coagulometer from Diagnostica Stago; if using a different instrument, please follow operation guidelines recommended by the instrument manufacturer.).
- 6.4.4 Add one metal ball into each cuvette and allow to warm for at least 3 minutes before use.
- 6.4.5 Add 100 µL of control plasma (step 5.5.8) or test plasma (step 5.5.9) to a cuvette. Prepare 1 cuvettes (1 strip, 4 wells) for each plasma sample.
- 6.4.6 Start timer for each of the test rows by pressing A, B, C or D buttons. Ten seconds before time is up, timer starts beeping. When this happens, immediately transfer cuvettes to PIP row and press PIP button to activate pipettor.
- 6.4.7 When time is up, add 100 µL of Neoplastin reagent to control plasma samples or 100 µL of cell suspension from step 6.3.6 in lieu of coagulation activation reagent to corresponding cuvettes, and record coagulation time.

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6.5 Flow chart

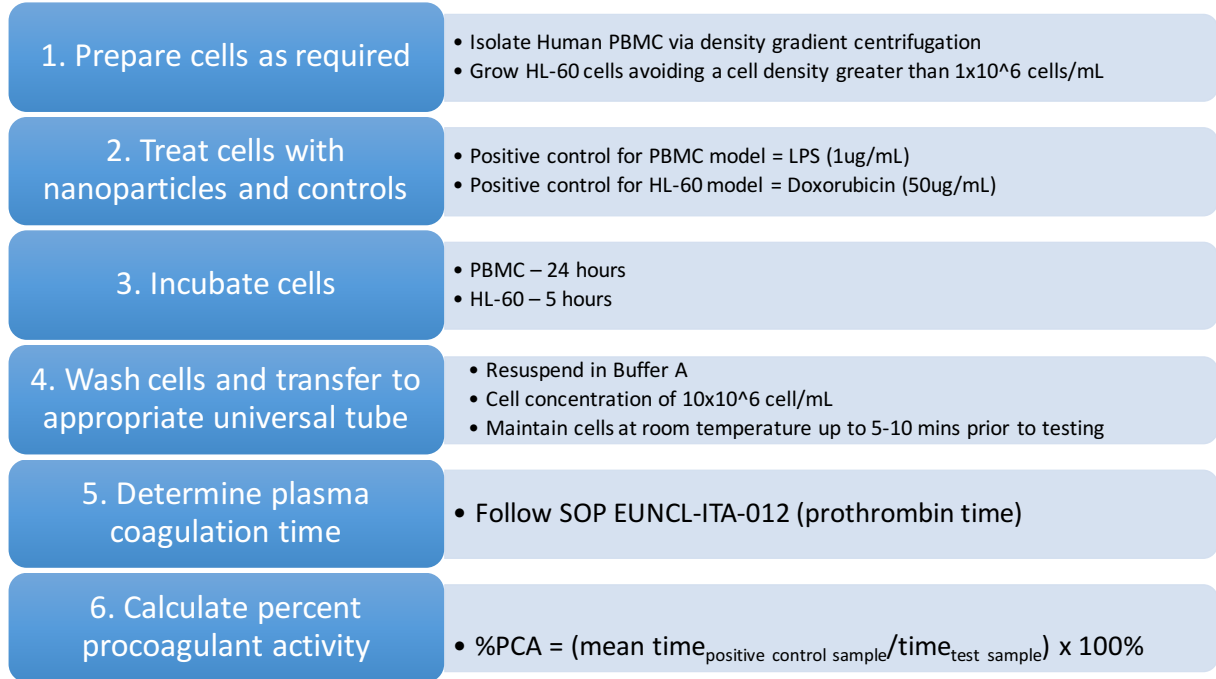


Figure 1:
Brief outline of the workflow.

6.6 Calculations and Interpretation

6.6.1 A percent coefficient of variation should be calculated for each control or test sample according to the following formula:

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

6.6.2 A percent procoagulant activity of a nanoparticle test sample is calculated according to the following formula:

$$\%PCA = (\text{mean time}_{\text{positive control sample}} / \text{time}_{\text{test sample}}) \times 100\%$$

7 Quality Control, Quality Assurance, Acceptance Criteria

7.1 %CV between replicates representing individual wells in the cuvette should be within 5%. This limit is stored in the instrument setting. When %CV is greater than 5, you will see a * mark on the print out.

7.2 % CV between replicates of test plasma samples should be within 25%.

7.3 If two duplicates of the same study sample demonstrated results >25% different, this test sample should be re-analyzed.

7.4 Positive control is considered positive if coagulation time in this sample is less than 360 sec. Coagulation time induced by HL-60 cells treated with doxorubicin and that

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induced by PBMC treated with LPS may vary from passage to passage and from donor to donor, respectively. Typical coagulation times observed in our lab with HL-60 cells treated with 50 µg/mL doxorubicin is 60-90 seconds. Typical coagulation times observed in our lab with PBMC treated with 1 µg/mL LPS is 200-300 seconds; that induced by ionophore is about 150 seconds.

8 Health and Safety Warnings, Cautions and Waste Treatment

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

APL	acute promyelocytic leukemia
CV	coefficient of variation
DIC	disseminated intravascular coagulation
FBS	fetal bovine serum
HBSS	Hank's balanced salt solution
LPS	lipopolysaccharide
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCA	procoagulant activity
RPMI	Roswell Park Memorial Institute
RT	room temperature
SD	standard deviation
U	units

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10 References

1. Barbui T, Falanga A: Disseminated intravascular coagulation in acute leukemia. *Semin Thromb Hemost.* 27(6), 593-604 (2001).
2. Franchini M, Dario Di Minno MN, Coppola A: Disseminated intravascular coagulation in hematologic malignancies. *Semin Thromb Hemost.* 36(4), 388-403 (2010).
3. Higuchi T, Toyama D, Hirota Y *et al.*: Disseminated intravascular coagulation complicating acute lymphoblastic leukemia: a study of childhood and adult cases. *Leuk Lymphoma.* 46(8), 1169-1176 (2005).
4. Levi M: Cancer and DIC. *Haemostasis.* 31 Suppl 1, 47-48 (2001).
5. Levi M: Disseminated intravascular coagulation in cancer patients. *Best Pract Res Clin Haematol.* 22(1), 129-136 (2009).
6. Uchiumi H, Matsushima T, Yamane A *et al.*: Prevalence and clinical characteristics of acute myeloid leukemia associated with disseminated intravascular coagulation. *Int J Hematol.* 86(2), 137-142 (2007).
7. Napoleone E, Zurlo F, Latella MC *et al.*: Paclitaxel downregulates tissue factor in cancer and host tumour-associated cells. *Eur J Cancer.* 45(3), 470-477 (2009).
8. Swystun LL, Shin LY, Beaudin S, Liaw PC: Chemotherapeutic agents doxorubicin and epirubicin induce a procoagulant phenotype on endothelial cells and blood monocytes. *J Thromb Haemost.* 7(4), 619-626 (2009).
9. Khemani RG, Bart RD, Alonzo TA *et al.*: Disseminated intravascular coagulation score is associated with mortality for children with shock. *Intensive Care Med.* 35(2), 327-333 (2009).
10. Lando PA, Edgington TS: Lymphoid procoagulant response to bacterial endotoxin in the rat. *Infect Immun.* 50(3), 660-666 (1985).
11. Oh D, Jang MJ, Lee SJ *et al.*: Evaluation of modified non-overt DIC criteria on the prediction of poor outcome in patients with sepsis. *Thromb Res.* 126(1), 18-23 (2010).

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12. Slofstra SH, ten Cate H, Spek CA: Low dose endotoxin priming is accountable for coagulation abnormalities and organ damage observed in the Shwartzman reaction. A comparison between a single-dose endotoxemia model and a double-hit endotoxin-induced Shwartzman reaction. *Thromb J.* 4, 13 (2006).
13. Fibach E, Treves A, Korenberg A, Rachmilewitz EA: In vitro generation of procoagulant activity by leukemic promyelocytes in response to cytotoxic drugs. *Am J Hematol.* 20(3), 257-265 (1985).
14. Gralnick HR, Abrell E: Studies of the procoagulant and fibrinolytic activity of promyelocytes in acute promyelocytic leukaemia. *Br J Haematol.* 24(1), 89-99 (1973).
15. Hiller E, Saal JG, Ostendorf P, Griffiths GW: The procoagulant activity of human granulocytes, lymphocytes and monocytes stimulated by endotoxin. Coagulation and electron microscopic studies. *Klin Wochenschr.* 55(15), 751-757 (1977).
16. Kwaan HC, Wang J, Boggio LN: Abnormalities in hemostasis in acute promyelocytic leukemia. *Hematol Oncol.* 20(1), 33-41 (2002).
17. Stein E, McMahon B, Kwaan H *et al.*: The coagulopathy of acute promyelocytic leukaemia revisited. *Best Pract Res Clin Haematol.* 22(1), 153-163 (2009).
18. ten Cate H, Falanga A: Overview of the postulated mechanisms linking cancer and thrombosis. *Pathophysiol Haemost Thromb.* 36(3-4), 122-130 (2008).
19. ten Cate H, Falanga A: The pathophysiology of cancer and thrombosis. Summary and conclusions. *Pathophysiol Haemost Thromb.* 36(3-4), 212-214 (2008).
20. Walsh J, Wheeler HR, Geczy CL: Modulation of tissue factor on human monocytes by cisplatin and adriamycin. *Br J Haematol.* 81(4), 480-488 (1992).
21. Wheeler HR, Geczy CL: Induction of macrophage procoagulant expression by cisplatin, daunorubicin and doxorubicin. *Int J Cancer.* 46(4), 626-632 (1990).
22. Dobrovolskaia MA, McNeil SE. Understanding the correlation between in vitro and in vivo immunotoxicity tests for nanomedicines. *J Control Release.* 2013 Dec10;172(2):456-66

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