NCL Method ITA-1
Version 1.0

Analysis of Hemolytic Properties of Nanoparticles

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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.

Method is written by: ______________________ 16 Dec 2005

Marina A. Dobrovolskaia, Ph.D. Date

______________________ 12-16-05 .

Barry W. Neun, B.S. Date

Method validation was conducted on: 5/2/2005 – 6/6/2005 .

Date

Validation report is complete on: 6/17/2005 .

Date

Testing facility: NCL, NCI-Frederick, Bldg. 469, Room 250 .

1. Introduction

This document describes a protocol for quantitative colorimetric determination of hemoglobin in whole blood (total blood hemoglobin - TBH) and hemoglobin released into plasma (plasma free hemoglobin – PFH) when blood is exposed to nanoparticles. Hemoglobin and its derivatives, except sulfhemoglobin, are oxidized to methemoglobin by ferricyanide in the presence of alkali. Cyanmethemoglobin is then formed from the methemoglobin by its reaction with cyanide (Drabkin's solution). The cyanmethemoglobin can be then detected by spectrophotometer set at 540 nm. The hemoglobin standard is used to build a standard curve covering the concentration range from 0.025 to 0.80 mg/mL and to prepare quality control samples at low (0.0625mg/mL), mid (0.125 mg/mL) and high (0.625mg/mL) concentrations to monitor assay performance. Required sample volume is 300 μL, i.e. 100 μL per test-replicate.

The results expressed as percent of hemolysis are used to evaluate the acute *in vitro* hemolytic properties of nanoparticles**.**

2. Reagents

2.1. Cyanmethemoglobin (CMH) reagent, StanBio, cat. # 0321-380

2.2. Hemoglobin Standard, StanBio, cat. # 0325-006

2.3. Ca/Mg free DPBS, Sigma, cat # D8537, store at RT

2.4. Pooled normal human whole blood anti-coagulated with Li-heparin

2.5. Poly-L-Lysine hydrobromide, MW 150 000 – 300 000, Sigma, cat#P1399

2.6. Polyethylene glycol, av. MW 8 000, Sigma cat# P1458

2.7. Distilled water

Note: Equivalent reagents from other vendor can be used

3. Equipment

3.1. Pipettes covering range from 0.05 to 10 mL

3.2. 96 well plates

3.3. Water bath set at 37 °C

3.4. Plate reader

3.5. Plastic beakers

3.6. Polypropylene tubes 15 mL

3.7. Centrifuge set at 700-800g

4. Preparation of Standards and Controls.

4.1 Preparation of Calibration Standards

Example is shown in the table below. Volumes can be adjusted based on the need.

Level	Nominal Conc.,mg/mL	Preparation Procedure
Cal 1	0.80	2 mL of stock solution
Cal 2	0.40	1mL Cal1 + 1 mL CMH reagent
Cal 3	0.20	1mL Cal2 + 1 mL CMH reagent
Cal 4	0.10	1mL Cal3 + 1 mL CMH reagent
Cal 5	0.05	1mL Cal4 + 1 mL CMH reagent
Cal 6	0.025	1mL Cal5 + 1 mL CMH reagent

4.2. Preparation of Quality Controls

Example is shown in the table below. Volumes can be adjusted based on the need.

Level	Nominal Conc.,mg/mL	Preparation Procedure
QC 1	0.625	1.5 mL of stock solution + 0.42 mL CMH reagent
QC 2	0.125	200 µL QC1 + 800 µL CMH reagent
QC 3	0.0625	100 µL QC1 + 900 µL CMH reagent

4.3. Preparation of Positive Control

Dissolve poly-L-Lysine powder to a final concentration of 1% (10mg/mL) in sterile distilled water. Prepare daily use aliquots and store at a nominal temperature of -20 °C. Alternatively, 1% TritonX-100 in water can be used as a positive control. PLL results in 30±5% hemolysis. Triton X-100 results in 90±5% hemolysis.

4.4. Preparation of Negative Control

Polyethylene glycol is supplied as 40% stock solution in water. Use this solution as the negative control. Store the stock solution at a nominal temperature of +4 °C.

5. Preparation of Study Samples

For the initial screen the test concentration is selected based on results from general toxicity assays. A nanomaterial, which revealed toxicity in general toxicity assay, is tested at two

concentrations selected at the low and the high end of the dose response curve. A nanomaterial, which did not reveal toxicity in a general toxicity assays is tested at one concentration equal to highest dose tested in general toxicity assay. The assay requires 300 μL of test material. Nanomaterial and buffer used for its storage/reconstitution should be tested in the same assay. Respectively, 300 μL of the buffer is required.

6. Experimental Procedure

6.1. Collect whole blood in tubes containing Li-heparin as anti coagulant from at least three donors. The blood can be stored at 2-8°C for up to 48 h. On the day of assay prepare pooled blood by mixing equal proportion of blood from each donor.

6.2. Take 2-3 mL aliquot of the pooled blood and centrifuge 15 min at 800g.

6.3. Collect supernatant. Keep at room temperature while preparing standard curve, quality controls and total hemoglobin sample. The collected sample is used to determine plasma free hemoglobin (PFH).

6.4. Add 200 μL of each calibration standard, quality control and blank cyanmethemoglobin (CMH) reagent per well on 96 well plate. Fill 2 wells for each calibrator and 4 wells for each quality control (QC) and blank. Position test samples so as they are bracketed by QC.

6.5. Add 200 μL of total blood hemoglobin (TBH) sample prepared by combining 20μL of the pooled whole blood and 5.0 mL of cyanmethemoglobin reagent. Fill 6 wells.

6.6. Add 100 μL of plasma per well on 96 well plate. Fill 6 wells.

6.7. Add 100 μL of cyanmethemoglobin reagent to each well containing sample.
Note: <u>Do not</u> add cyanmethemoglobin reagent to wells containing calibration standards and quality controls.

6.8. Cover plate with plate sealer and gently shake on a plate shaker for 1-2 minutes (shaker speed settings should be vigorous enough to allow mixing the reagent, but to avoid spillage and cross-well contamination; e.g. LabLine shaker speed 2-3).

6.9. Read the absorbance at 540 nm to determine hemoglobin concentration. Remember to use the dilution factor 2 for PFH sample and dilution factor 251 for TBH. If calculated PFH concentration is below 1 mg/mL proceed to the next step.

6.10. Dilute pooled whole blood with Ca^{2+}/Mg^{2+} free DPBS to adjust total hemoglobin concentration to 10± 2 mg/mL.

6.11. In an eppendorf tube add 100 µL of sample, blank (i.e. buffer used to reconstitute test sample), positive or negative control. Prepare 6 tubes for each unknown sample; 3 tubes for the blank, 2 tubes for the positive control and 2 tubes for the negative control.

Note: if sample volume is below 100 µL adjust volume with Ca^{2+}/Mg^{2+} free DPBS.

6.12. Add 700 µL of Ca^{2+}/Mg^{2+} free DPBS to each tube.

6.13. Add 100 µL of the whole blood prepared in step 6.10 to each tube, except for 3 tubes of each test sample. In these tubes add 100 µL of Ca^{2+}/Mg^{2+} free DPBS. These samples represent a "minus blood" control and are used to evaluate potential interference of nanomaterial with the assay (e.g. absorbance at or close to 540 nm, reactivity with CMH reagent etc.)

6.14. Cover tubes and gently rotate to mix.

Note: vortexing may damage erythrocytes and should be avoided.

6.15. Place the tubes in a water bath set at 37 °C and incubate for 3hours ±15 min mixing the samples every 30 min. alternatively, tubes may be incubated on a tube rotator in an incubator set at 37 °C.

6.16. Remove the tubes from water bath or incubator. If water bath was used dry an excess of water with absorbent paper.

6.17. Centrifuge the tubes for 15 min at 800g.

Note: when centrifugation is complete examine tubes and record any unusual appearance that can help in result interpretation. See example in section 9 of the method.

***Important**: If nanoparticles have absorbance at or close to 540nm, removal of these particles from supernatant will be required before proceeding to the next step. For example, 10-50 nm colloidal gold nanoparticles have absorbance at 535nm. After step 6.17 supernatants should be transferred to fresh tubes and centrifuged 30 min at 18 000 g. Method of nanoparticles removal from supernatant is nanoparticles specific, and when applied appropriate validation experiments should be conducted to ensure that a given separation procedure does not affect assay performance. In certain cases removal of particles is not feasible. When this is the case, assay result obtained for a particle*

incubated with blood is adjusted by subtracting result obtained for the same particle in "minus blood" control (see section 6.13).

6.18. Prepare fresh set of calibrators and quality controls.

6.19. To a fresh 96 well plate add 200 µL of blank reagent, calibrators, quality controls or total blood hemoglobin sample (TBHd) prepared by combining 400 µL of blood from step 6.10 with 5.0 mL of CMH reagent. Fill 2 wells for each calibrator, 4 wells for blank and each quality control, and 6 wells for TBHd sample. As before position all test samples between quality controls on the plate.

6.20. Add 100 µL per well of test samples, positive and negative controls prepared in step 6.17. Fill 12 wells for each sample (2 wells from each of six tubes prepared in step 6.11) and 4 wells for each control (2 wells from each of two tubes).

6.21. Add 100 µL of cyanmethemoglobin reagent to each well containing sample and controls.

Note: Do not add cyanmethemoglobin reagent to wells containing calibration standards, quality controls and TBHd.

6.22. Cover plate with plate sealer and gently shake on a plate shaker (LabLine shaker speed settings 2-3 or as appropriate for a given shaker).

6.23. Read the absorbance at 540 nm to determine concentration of hemoglobin.
Remember to use the dilution factor 16 for samples and controls and dilution factor 13.5 for TBHd.

7. Calculations

Four-parameter regression algorithm is used to build calibration curve. The following parameters should be calculated for each calibrator and quality control sample:

Percent Coefficient of Variation**:** %CV=SD/Mean x 100%

Percent Difference From Theoretical:

$$PDFT = \frac{\text{Calculated Concentration} - \text{Theoretical Concentration}}{\text{Theoretical Concentration}} \times 100\%$$

%CV should be calculated for each blank, positive control, negative control and unknown sample.

8. Acceptance Criteria

8.1. %CV and PDFT for each calibration standard and quality control should be within 20%. The exception is Cal 6, for which 30 % is acceptable. A plate is accepted if 2/3 of all QC levels and at least one of each level have demonstrated acceptable performance. If not entire run should be repeated.

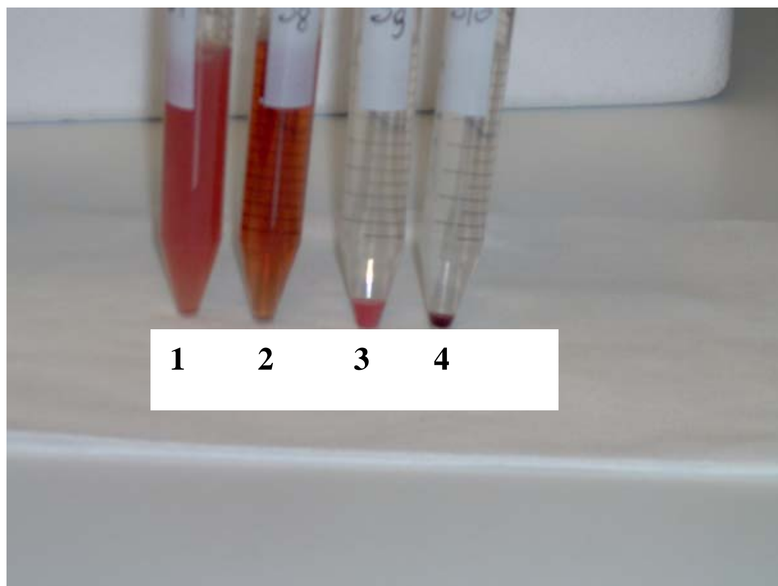
8.2. % CV for each positive control, negative control and unknown sample should be within 20 %. At least one replicate of positive and negative control should be acceptable for run to be accepted.

8.3. If both replicates of positive control or negative control fail to meet acceptance criterion described in 8.2 the run should be repeated.

8.4. Within the acceptable run if two of three replicates of unknown sample fail to meet acceptance criterion described in 8.2 this unknown sample should be re-analyzed.

9. Example of Sample appearance.

This example demonstrates the importance of recording sample appearance after centrifugation to avoid false negative results.



On the picture shown above polystyrene nanoparticles with size 20 nm (tube 1) and polystyrene nanoparticles with size 50 nm (tube 2) demonstrated hemolytic activity which can be observed by the color of supernatant. Polystyrene nanoparticles with size 80nm were also hemolytic; however they absorbed hemoglobin that can be determined by the pellet size and color. Supernatant of this sample is used in assay and absorbance at 540 nm will demonstrate negative

result. Sample #4 is the negative control. No hemolytic activity was observed in supernatant, and intact red blood cells formed tight dark red pellet on the bottom of the tube.

10. References

10.1. ASTM standard practice F 756-00. Assessment of hemolytic properties of materials.

10.2. DeSilva B, Smith W, Weiner R, Kelley M, Smolec J, Lee B, Khan M, Tacey R, Hill H, Celniker A. Recommendations for the bioanalytical method validation of ligand-binding assays to support pharmacokinetic assessments of macromolecules. Pharm Res. 2003 Nov; 20(11):1885-900.

10.3. Bioanalytical method validation. Guidance for industry. FDA/CDER/CVM. May 2001. BP.