Method for Analysis of Nanoparticle Hemolytic Properties *In Vitro*

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Supplementary Materials

Reagents

Cyanmethemoglobin (CMH) reagent and Hemoglobin standard were purchased from StanBio. Ca^{2+}/Mg^{2+} free DPBS, Polyethylene glycol (av. MW 8 000), and Poly-L-Lysine (PLL) hydrobromide (MW 150 000 – 300 000) were from Sigma-Aldrich. Polystyrene nanoparticles with size of 20, 50 and 80nm were purchased from Duke Scientific Corporation. Colloidal gold nanoparticles with size of 5, 10, 20, 30, 40 and 50nm were from TedPella Inc. G5 and G6 PAMAM dendrimers with amine, carboxy and hydroxyl surface were Dendritic Nanotechnologies Inc. Nanoliposomes were kindly provided by Dr. Mark Kester (Pennsylvania State University). Nanoemulsions were kindly provided by Dr.Mansoor Amiji (Northeastern University). Triazine dendrimers and water-soluble fullerene derivatives (C3) were kindly provided by Dr. Eric Simanek (Texas A&M University) and C60 Inc., respectively. Abraxane, Propofol and Doxil were purchased through NIH pharmacy.

Research Donor Blood.

Healthy volunteer blood specimens were drawn under NCI-Frederick Protocol OH99-C-N046. Blood was collected in BD vacutainer tubes containing Lithium heparin (hemolsyis test) or sodium citrate (platelet aggregation test) as anticoagulant. Specimens from at least three donors were pooled.

Assay procedure

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

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

Preparation of Positive Control

Dilute Triton X-100 in water to have final concentration of 10% v/v.

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**.**

Preparation of Study Samples

The assay requires 600 μL of test material. Nanomaterial and buffer used for its storage/reconstitution should be tested in the same assay. Respectively, 600 μL of the buffer is required. Concentration of nanomaterials depends on material's solubility and stability in aqueous solutions. The materials should be tested at four concentration. The highest concentration is the one reasonably achievable based on nanoparticle availability and stability. This concentration is referred to as to "stock". Three serial 1 to 5 dilutions of the stock are prepared in PBS or other physiological buffer and included in the analysis.

Experimental procedure

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.
2. Take 2-3 mL aliquot of the pooled blood and centrifuge 15 min at 800g.
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).
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.
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. Add 100 μL of plasma per well on 96 well plate. Fill 6 wells.
7. Add 100 μL of cyanmethemoglobin reagent to each well containing sample.

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

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).
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.
10. Dilute pooled whole blood with Ca^{2+}/Mg^{2+} free DPBS to adjust total hemoglobin concentration to 10± 2 mg/mL.
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.
12. Add 700 µL of Ca^{2+}/Mg^{2+} free DPBS to each tube.
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.)
14. Cover tubes and gently rotate to mix.
    Note: vortexing may damage erythrocytes and should be avoided.
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.
16. Remove the tubes from water bath or incubator. If water bath was used dry an excess of water with absorbent paper.
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).*

18. Prepare fresh set of calibrators and quality controls.
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.
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).
21. Add 100 µL of cyanmethemoglobin reagent to each well containing sample and controls.

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

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).
23. Read the absorbance at 540 nm to determine concentration of hemoglobin. Remember to use the dilution factor 18 for samples and controls and dilution factor 13.5 for TBHd.

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:

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

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

Assay Acceptance Criteria

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