Project:

Analysis of Haemolytic Properties of Nanoparticles

AUTHORED BY:	DATE:
Neill Liptrott	10.02.2016

REVIEWED BY:	DATE:
Matthias Roesslein	18.02.2016
Matthias Roesslein (1.3)	30.03.2017

APPROVED BY:	DATE:
Matthias Roesslein	21.02.2016
Matthias Roesslein	30.03.2017

DOCUMENT HISTORY

Effective Date	Date Revision Required	Supersedes
DD/MM/YYYY	DD/MM/YYYY	DD/MM/YYYY

Version	Approval Date	Description of the Change		Author / Changed by
1.0	10.02.2016	All	Initial Document	Neill Liptrott
1.3	30.03.2017	All	Small corrections and adoptions	Neill Liptrott

Table of Content

1 Introduction

Erythrocytes comprise approximately 45% of whole blood by volume. Hemolysis refers to the damage of red blood cells leading to the release of erythrocyte intracellular content into blood plasma. When it occurs *in vivo* hemolysis can lead to anemia, jaundice and other pathological conditions, which may become life threatening. Hemoglobin is a dominant protein carried by erythrocytes. When it is contained inside the cell it plays a key role in carrying oxygen to other cells and tissues. However, extracellular hemoglobin is toxic and may affect vascular, myocardial, renal and central nervous system tissues. This is why all medical devices and drugs which come in contact with blood are required to be tested for potential hemolytic properties.

2 Principle of the Method

This document describes a protocol for quantitative colorimetric determination of total hemoglobin in whole blood (TBH) and plasma free hemoglobin (PFH). An increase in the plasma free hemoglobin is indicative of erythrocyte damage by the test material (a positive control substance or a nanoparticle). Hemoglobin, released from damaged erythrocytes, is unstable and forms several derivatives with difference optical properties. Hemogblobin and its derivatives, except sulfhemoglobin, are oxidized to methemoglobin by ferricyanide in the presence of alkali. Addition of the Drabkin's solution containing cyanide (also called CMH Reagent) converts methemoglobin into CMH form, which is the most stable form of hemoglobin and can then be detected by spectrophotometry at 540 nm. Addition of CMH Reagent to the whole blood sample is needed to lyse erythrocytes and estimate TBH, while its addition to plasma is used to detect PFH. A 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 (0.0625 mg/mL), mid (0.125 mg/mL) and high (0.625mg/mL) concentrations for monitoring assay performance. The results, expressed as percent of hemolysis, are used to evaluate the acute *in vitro* hemolytic properties of nanoparticles**.** Other versions of the hemolysis assay are available in the literature; these protocols omit reduction of the hemoglobin to its stable CMH form and estimate the amount of hemolysis by measuring oxyhemoglobin at one of its primary absorbance peaks (i.e. 415, 541 or 577nm). These assays have been previously reviewed by Malinauskas R.A. [1]. The protocol described in this document is based on ASTM International standards [2, 3].

3 Applicability and Limitations (Scope)

This assay is for the determination of the haemolytic properties of nanoparticles. The cause of this haemolysis cannot be directly determined and must be investigated by subsequent assays. Care must be taken when analyzing nanoparticles that may interfere with the assay by absorbing haemoglobin onto (false negative) them or by having optical properties which may interfere with the assay measurement (false positive). Examples of these are to be found in section 6.1

4 Related Documents

Table 1:

Document ID	Document Title

5 Equipment and Reagents

5.1 Equipment

5.1.1 Water bath set at 37ºC or incubator set at 37ºC with a tube rotator

5.1.2 Plate reader capable of reading absorbance at 540 nm

5.1.3 Centrifuge capable of running at 800 x g and suitable for vacutainers or larger tubes

5.1.4 Centrifuge capable of running at 800 x g and 18,000 x g, and suitable for microcentrifuge tubes

5.2 Materials

5.2.1 Pipettes covering the range 0.05 mL to 10.0 mL

5.2.2 96 well plates suitable for cell culture

5.2.3 Polypropylene tubes, 15 mL

5.2.4 Microcentrifuge tubes, 1.5 mL

5.3 Reagents

5.3.1 Cyanmethemoglobin (CMH) Reagent (Teco Diagnostics, Anahem, CA, H526-480)

5.3.2 Hemoglobin Standard (StanBio, 0325-006)

5.3.3 Ca2+/Mg2+ free PBS, (GE Life Sciences, SH30256.01)

5.3.4 Normal human whole blood anti-coagulated with Li-heparin from at least 3 donors

5.3.5 Triton X-100 (Sigma, 93443)

5.4 Preparation of Standards and Controls

5.4.1 Preparation of Calibration Standards

An example of the preparation of standards is shown in Table 1. Volumes can be adjusted as needed. Prepare fresh standards for each experiment, discard leftovers after use.

Table 1. Preparation of Calibration Standards

Standard	Nominal Concentration (mg/mL)	Preparation Procedure
Cal 1	0.80	2 mL of stock solution
Cal 2	0.40	1 mL Cal 1 + 1 mL CMH reagent
Cal 3	0.20	1 mL Cal 2 + 1 mL CMH reagent
Cal 4	0.10	1 mL Cal 3 + 1 mL CMH reagent
Cal 5	0.05	1 mL Cal 4 + 1 mL CMH reagent
Cal 6	0.025	1 mL Cal 5 + 1 mL CMH reagent

5.4.2 Preparation of Quality Controls

An example of the preparation of QC standards samples is shown in Table 2. Volumes can be adjusted as needed. Prepare fresh QC for each experiment, discard leftovers after use.

Table 2. Preparation of Quality Control Standards

Standard	Nominal Concentration (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 QC 1 + 800 µL CMH reagent
QC 3	0.0625	100 µL QC 1 + 900 µL CMH reagent

5.4.3 Preparation of Positive Control

Any reagent or a nanomaterial, which reproducibly induces ≥8% of hemolysis in this assay can be used as the assay positive control.

Triton X-100 at a stock concentration of 1% (10mg/mL) is an example positive control. Triton X-100 can be prepared in sterile distilled water and kept refrigerated (nominal temperature of 4 °C) for up to 2 weeks. Alternatively, a commercial 10% solution can be used and stored according to the manufacturer's instructions.

5.4.4 Preparation of Negative Control

Phosphate Buffered Saline is supplied as sterile solution and can be used as the negative control. Store the stock solution at room temperature. Alternatively, a solution of polyethylene glycol or any other material known not to be hemolytic can be used as the negative control. When such reagents are used, please refer to the preparation and storage instructions by the reagent manufacturer.

5.4.5 Preparation of 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.

5.4.6 Preparation of Inhibition/Enhancement Control

This control is needed to estimate potential interaction between nanoparticles and plasma free hemoglobin which masks hemoglobin from detection by the assay. The control is prepared by spiking cell-free supernatant obtained from the positive control sample with nanoparticles at the concentrations matching those analyzed by the assay. For example, if a nanoparticle is tested at four concentrations (1, 0.2, 0.04 and 0.008 mg/mL), then cell-free supernatant derived from the assay positive control should be spiked with 1, 0.2, 0.04 and 0.008 mg/mL of that nanoparticle. This control is helpful in identifying false-negative results when a material with strong hemolytic potential (i.e. % hemolysis >90) is used as the assay positive control. It also helps identifying potential enhancement type of interference when a low potency positive control (% hemolysis 8-50) is used. Dilution factor 1.1 is used to adjust the test results derived from these samples to account for the PC dilution.

False positive interference resulting from nanoparticle optical properties overlapping with the assay wavelength (540 nm) is identified by nanoparticle only blood free control (see section 4.7).

5.4.7 Preparation of Blood Free Control

Nanoparticles diluted in PBS to the same final concentration as those evaluated in the assay using whole blood and subjected to the same manipulation as test samples (i.e. incubation at 37C for 3 hours, followed by centrifugation and mixing with CMH reagent) can serve as additional control to rule out false-positive assay results.

5.5 Preparation of Blood and Study Samples

5.5.1 Test sample preparation

This assay requires 1.0 mL of nanoparticle solution, at a concentration 9 X the highest final tested concentration, dissolved/resuspended in PBS. 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 (4) 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.

$$human\ dose = \frac{mouse\ dose}{12.3} = \frac{123\frac{mg}{kg}}{12.3} = 10\ mg/kg$$ (see reference [5])

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.

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

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

The assay will evaluate 4 concentrations: 10 X (or when feasible 100X, 30X or 5X) of 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 18 mg/mL will be prepared and diluted 10-fold (1.8mg/mL), followed by two 1:5 serial dilutions (0.36 and 0.072 mg/mL). When 0.1 mL of each of these samples is added to the test tube and mixed with

0.7 mL of PBS and 0.1 mL of blood, the final nanoparticle concentrations tested in the assay are: 2.0, 0.2, 0.04 and 0.008 mg/mL.

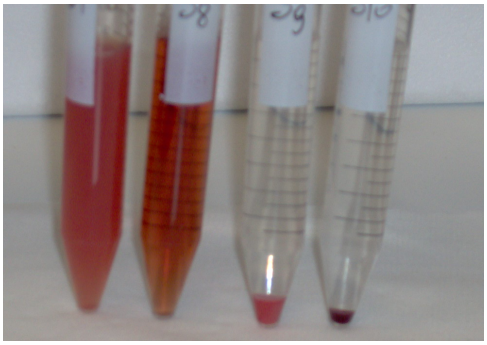
5.5.2 Blood preparation

Collect whole blood in tubes containing Li-heparin as an anti-coagulant from at least three donors. Discard first 10 cc. The blood can be used fresh or stored at 2-8°C for up to 48 h. On the day of the assay, prepare pooled blood by mixing equal proportions of blood from each donor. Donors are preselected so that compatible blood types are mixed. The assay can also be performed in blood of individual donors.

6 Procedure

6.1 General remarks

It is important to record any unusual appearance of samples as this may assist in interpreting the data. An example is given below in figure 1 which demonstrates the importance of recording sample appearance after centrifugation to avoid false negative results.



1 2 3 4

Figure 1. Example of Nanoparticle Interference and ways of handling. On the picture shown above, polystyrene nanoparticles with a size of 20 nm (tube 1) and polystyrene nanoparticles with a size of 50 nm (tube 2) demonstrated hemolytic activity which can be observed by the color of supernatant. Polystyrene nanoparticles with a size of 80 nm (tube 3) were also hemolytic. However, they absorbed hemoglobin; this can be determined by the pellet size and color. Supernatant of this sample used in assay and measured at 540 nm will demonstrate negative result. Tube 4 is the negative control. No hemolytic activity was observed in the supernatant, and intact red blood cells formed a tight dark red pellet on the bottom of the tube.

If nanoparticles have absorbance at or close to 540 nm, 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 535 nm. After step 6.18, supernatants should be transferred to fresh tubes and centrifuged 30 min at 18, 000 x g. Method of nanoparticle 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 results obtained for a particle incubated with blood is adjusted by subtracting results obtained for the same particle in "minus blood" control (see section 6.13 and refer to samples in Rack 2). Examples of interference and ways to remove it are shown in Figures 2 and 3.

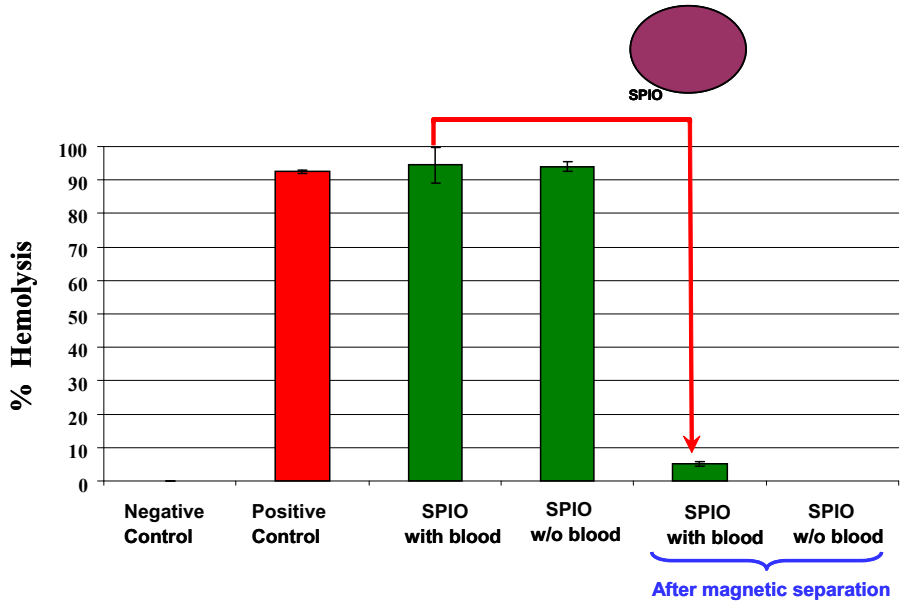


Figure 2. An example of a false-positive result due to nanoparticle absorbance at the assay wavelength. Due to the small size, the fullerene nanoparticle could not be removed from supernatant. Therefore, result adjustment was done to account for interference.

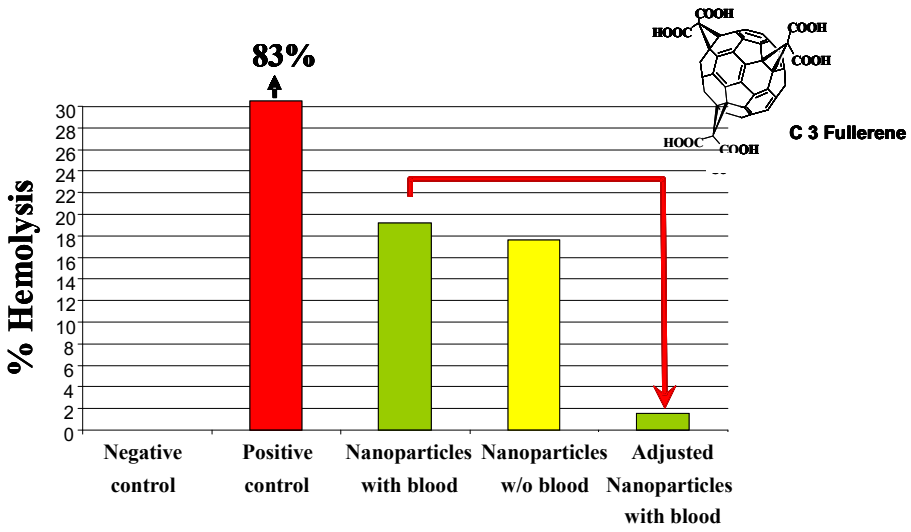


Figure 3. An example of a false-positive result due to nanoparticle absorbance at the assay wavelength. To remove iron oxide nanoparticles from supernatants, tubes containing supernatants were placed on magnets and incubated overnight at 4°C. Particles concentrated on the side of the tube adjacent to magnet, thus allowing removal of particle-free supernatant for analysis.

6.2 Experimental Procedure

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

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

6.2.3 Add 200 µL of each calibration standard, quality control, and blank (CMH Reagent) per well on a 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 (See Plate Map 1 Example in Appendix).

6.2.4 Add 200 µL of total blood hemoglobin (TBH) sample, prepared by combining 20 µL of the pooled whole blood and 5.0 mL of CMH Reagent. Fill 6 wells.

6.2.5 Add 100 µL of plasma (PFH) per well on 96 well plate. Fill 6 wells.

6.2.6 Add 100 µL of CMH Reagent to each well containing sample.

Important: <u>Do not</u> add cyanmethemoglobin CMH Reagent to wells containing calibration standards and quality controls.

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

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

6.2.9 Dilute pooled whole blood with Ca^{2+}/Mg^{2+} free PBS to adjust total blood hemoglobin concentration to 10 $\pm$ 2 mg/mL (TBHd).

6.2.10 Set up two racks. Rack 1 contains tubes for the sample incubation with blood. Rack 2 contains tubes for the nanoparticle only (no blood) control. Prepare 6 tubes for each test-sample and place 3 tubes into Rack 1 and 3 tubes into Rack 2. Place 4 tubes for the positive control, 2 tubes for the negative control and 2 tubes of the vehicle control (if the vehicle is PBS, this samples can be skipped) into Rack 1.

6.2.11 Add 100 µL of the test-sample or control in corresponding tubes in Rack 1 and Rack described in step 6.10.

6.2.12 Add 700 µL of Ca^{2+}/Mg^{2+} free PBS to each tube in Rack 1.

6.2.13 Add 800 µL of Ca^{2+}/Mg^{2+} free PBS into each tube in Rack 2.

6.2.14 Add 100 µL of the whole blood prepared in step 6.9 to all tubes in Rack 1.

6.2.15 Cover tubes and gently rotate to mix.

Note: vortexing may damage erythrocytes and should be avoided.

6.2.16 Place the tubes in a water bath set at 37ºC and incubate for 3 h ±15 min, mixing the samples every 30 min. Alternatively, tubes may be incubated on a tube rotator in an incubator set at 37˚C.

6.2.17 Remove the tubes from water bath or incubator. If a water bath was used, dry excess water with absorbent paper.

6.2.18 Centrifuge the tubes for 15 min at 800 x g.

Note: When centrifugation is complete examine tubes, and record any unusual appearance that can help in result interpretation. See example in Figure 1.

6.2.19 Prepare a fresh set of calibrators and quality controls.

6.2.20 Prepare inhibition enhancement controls by spiking positive control supernatant from step 6.18 with nanoparticles at the final particle concentration as in the test samples

6.2.21 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 6.9 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 (See Example Plate Map 2 in Appendix).

6.2.22 Add 100 μL per well of test samples and controls (positive, negative, and vehicle with and without blood) prepared in step 6.18 as well as IEC from step 6.20. Test each sample in duplicate.

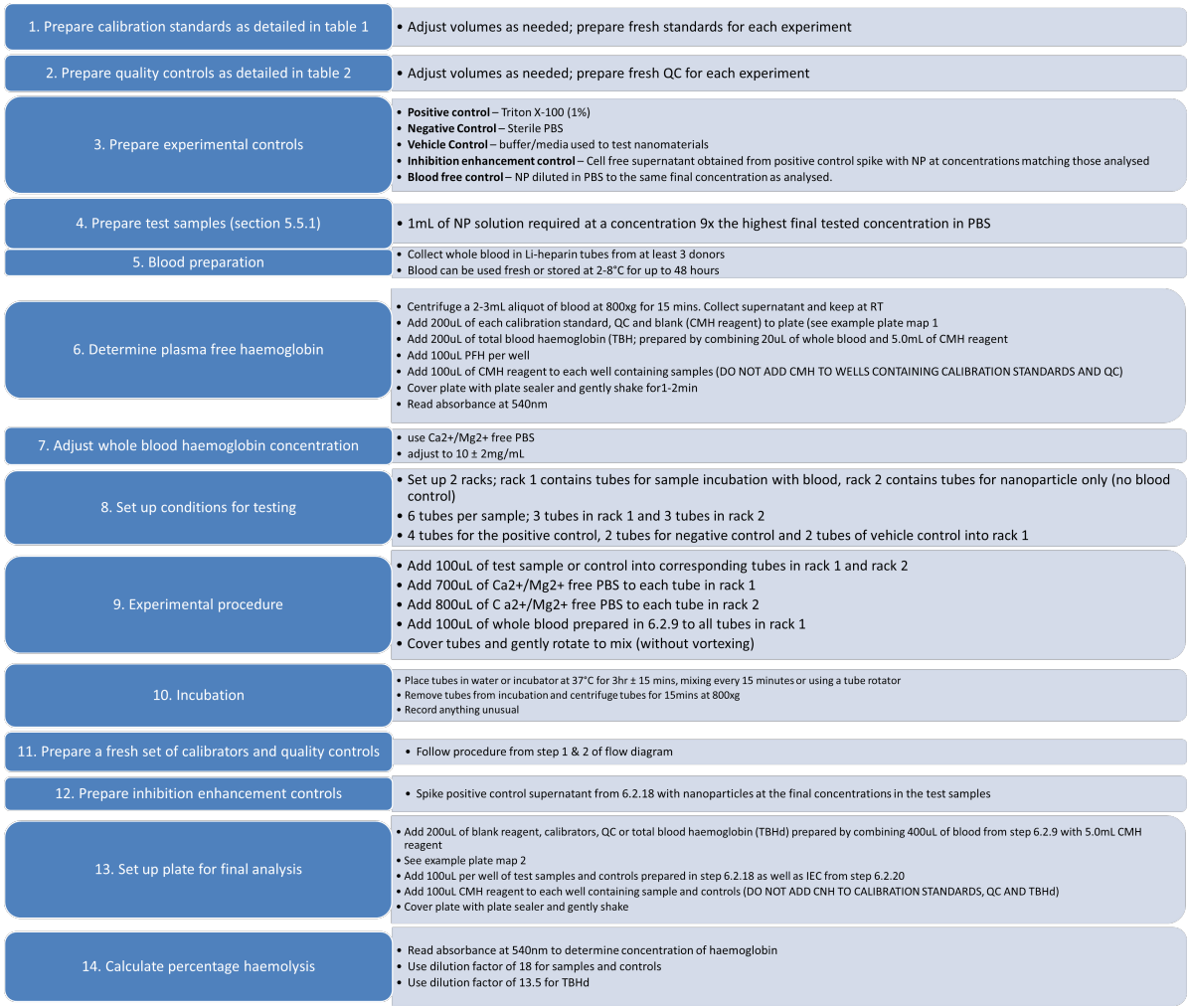
6.2.23 Add 100 μL of CMH Reagent to each well containing sample and controls.

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

6.2.24 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).

6.2.25 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.

6.3 Flow chart



6.4 Calculations and results interpretation

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

6.4.1 Percent Coefficient of Variation (%CV):

$$\frac{\text{Standard Deviation of a single value}}{\text{Mean}} \times 100\ \%$$

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

6.4.2 Percent Difference from Theoretical (PDFT):

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

6.4.3 Percent Hemolysis:

(Hemoglobin in test sample/TBHd) x 100 %

6.4.4. According to the references 2 and 3 percent hemolysis less than 2 means the test sample is not-hemolytic; 2-5 % hemolysis means the test sample is slightly hemolytic and > 5% hemolysis means the test sample is hemolytic.

7 Quality Control, Quality Assurance, Acceptance Criteria

7.1 %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.

7.2 % 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.

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

7.4 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.

8 Health and Safety Warnings, Cautions and Waste Treatment

8.1 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

API	active pharmaceutical ingredient
Cal	calibration
CMH	cyanmethemoglobin
CV	coefficient of variation
PBS	phosphate buffered saline
PFH	plasma free hemoglobin
QC	quality control
SPIO	super paramagnetic iron oxide
TBH	total blood hemoglobin
TBHd	total blood hemoglobin, diluted

10 Annex

Example Plate Map 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	QC 1	QC 2	QC 3	PFH	PFH
B	Blank	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	QC 1	QC 2	QC 3	PFH	PFH
C	PFH	TBH	TBH	TBH	Blank	QC 1	QC 2	QC 3				
D	PFH	TBH	TBH	TBH	Blank	QC 1	QC 2	QC 3				
E												
F												
G												
H												

Example Plate Map 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	QC 1	QC 2	QC 3	TS 1.0 mg/mL	TS 1.0 mg/mL
B	Blank	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	QC 1	QC 2	QC 3	TS 1.0 mg/mL	TS 1.0 mg/mL
C	NP 1.0 mg/mL	TS 0.2 mg/mL	TS 0.2 mg/mL	TS 0.2 mg/mL	TS 0.04 mg/mL	TS 0.04 mg/mL	TS 0.04 mg/mL	TS 0.008 mg/mL	TS 0.008 mg/mL	TS 0.008 mg/mL	TS (No Blood) 1.0 mg/mL	TS (No Blood) 1.0 mg/mL
D	TS 1.0 mg/mL	TS 0.2 mg/mL	TS 0.2 mg/mL	TS 0.2 mg/mL	TS 0.04 mg/mL	TS 0.04 mg/mL	TS 0.04 mg/mL	TS 0.008 mg/mL	TS 0.008 mg/mL	TS 0.008 mg/mL	TS (No Blood) 1.0 mg/mL	TS (No Blood) 1.0 mg/mL
E	TS (No Blood) 1.0 mg/mL	TS (No Blood) 0.2 mg/mL	TS (No Blood) 0.2 mg/mL	TS (No Blood) 0.2 mg/mL	TS (No Blood) 0.04 mg/mL	TS (No Blood) 0.04 mg/mL	TS (No Blood) 0.04 mg/mL	TS (No Blood) 0.008 mg/mL	TS (No Blood) 0.008 mg/mL	TS (No Blood) 0.008 mg/mL	PC	PC
F	TS (No Blood) 1.0 mg/mL	TS (No Blood) 0.2 mg/mL	TS (No Blood) 0.2 mg/mL	TS (No Blood) 0.2 mg/mL	TS (No Blood) 0.04 mg/mL	TS (No Blood) 0.04 mg/mL	TS (No Blood) 0.04 mg/mL	TS (No Blood) 0.008 mg/mL	TS (No Blood) 0.008 mg/mL	TS (No Blood) 0.008 mg/mL	PC	PC
G	NC	NC	TBHd	TBHd	TBHd	IEC1	IEC 2	IEC3	IEC4	QC 1	QC 2	QC 3
H	NC	NC	TBHd	TBHd	TBHd	IEC1	IEC 2	IEC3	IEC4	QC 1	QC 2	QC 3

IEC – inhibition enhancement control prepared by spiking PC supernatant with test nanoparticles; 1, 2, 3 and 4 refers to the concentration of the test nanoparticle.

11 References

1. Malinauskas, R.A., *Plasma hemoglobin measurement techniques for the in vitro evaluation of blood damage caused by medical devices.* Artif Organs, 1997. **21**(12): p. 1255-67.

2. 756-00, A.S.p.F., *Assessment of haemolytic properties of nanoparticles.*

3. E2524-08, A., *Test method for Analysis of Haemolytic Properties of Nanoparticles.* 2013.

4. Dobrovolskaia MA, McNeil SE. Understanding the correlation between in vitro and in vivo immunotoxicity tests for nanomedicines. J Control Release. 2013 Dec 10;172(2):456-66

5. CDER, Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers, 2005.