Designation: E2524 – 22

Standard Test Method for Analysis of Hemolytic Properties of Nanoparticles[1]

This standard is issued under the fixed designation E2524; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method covers assessing the effect of nanoparticulate materials on the integrity of red blood cells.

1.2 This test method uses diluted whole blood incubated with nanoparticulate material and the hemoglobin released from damaged red blood cells is determined.

1.3 This test method is similar to Practice F756 with the volumes reduced to accommodate nanoparticulate material.

1.4 This test method is part of the *in-vitro* preclinical characterization and is important for nanoparticulate material that will contact the blood in medical applications.

1.5 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.6 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.7 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

F748 Practice for Selecting Generic Biological Test Methods for Materials and Devices

F756 Practice for Assessment of Hemolytic Properties of Materials

F1877 Practice for Characterization of Particles

F1903 Practice for Testing for Cellular Responses to Particles ***in vitro***

2.2 *ISO Standard:*[3]

ISO 10993-4 Biological Evaluation of Medical Devices Part 4: Selection of Tests for Interactions with Blood

3. Terminology

3.1 *Acronyms:*

3.1.1 *Cal*—calibration standard

3.1.2 *CMH*—cyanmethemoglobin

3.1.3 *DPBS*—Dulbecco's phosphate-buffered saline

3.1.4 *PFH*—plasma-free hemoglobin

3.1.5 *QC*—quality controls

3.1.6 *TBH*—total blood hemoglobin

3.1.7 *TBHd*—blood sample diluted to 10 mg ± 1 mg/mL

4. Summary of Test Method

4.1 This test method describes a protocol for assessing acute *in-vitro* damage to red blood cells (that is, hemolysis) caused by exposure to nanoparticles.

4.2 This test method is based on the quantitative determination of hemoglobin released into PFH as a percentage of the TBH concentration when blood is exposed to nanoparticulate materials.

4.3 Using an established colorimetric assay,[4] hemoglobin and its derivatives, such as sulfhemoglobin, are oxidized to methemoglobin by ferricyanide in the presence of alkali. A stable CMH concentration is measured using a plate reader spectrophotometer set at 540 nm.

4.4 Hemoglobin standards are used to create a standard curve covering the range from 0.025 mg⁄mL to 0.8 mg/mL and prepare quality control samples at low (0.0625-mg/mL), mid (0.125-mg/mL), and high (0.625-mg/mL) concentrations to monitor assay performance. The required sample volume is 100 µL per test replicate.

[1] This test method is under the jurisdiction of ASTM Committee E56 on Nanotechnology and is the direct responsibility of Subcommittee E56.03 on Environment, Health, and Safety.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036, http://www.ansi.org.

[4] International Committee for Standardization in Haemotology, *Journal of Clinical Pathology*, Vol 31, 1978, pp. 139–143.

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

5. Significance and Use

5.1 This test method is one of a series of tests listed in Practice F748 and ISO 10993-4 to assess the biocompatibility of materials contacting blood in medical applications.

5.2 This test method is similar to Practice F756 but modified to accommodate nanoparticulate materials.

6. Apparatus

6.1 Pipettes covering range from 0.05 mL to 10 mL.

6.2 Ninety-six well plates.

6.3 Water bath set at 37 °C ± 1 °C.

6.4 Plate reader capable of measuring at 540 nm.

6.5 Plate shaker.

6.6 Plastic beakers.

6.7 Microcentrifuge tubes, 1.5 mL, translucent, not colored.

6.8 Centrifuge set at 700 *g* to 800 *g*.

7. Reagents

7.1 *Purity of Reagents*—Reagent-grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where such specifications are available.[5] Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

7.2 CMH reagent (also known as Drabkin's reagent).

7.3 Hemoglobin standard.

7.4 Ca^{2+}/Mg^{2+}-free DPBS.

7.5 Pooled normal human whole blood anticoagulated with Li-heparin.

7.6 Triton X-100.

7.7 Distilled water.

[5] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

TABLE 1 Calibration Standards

Level	Nominal Conc., mg/mL	Preparation Procedure
Cal 1	0.8	2 mL of stock solution
Cal 2	0.4	1 mL of Cal1 + 1 mL of CMH reagent
Cal 3	0.2	1 mL of Cal2 + 1 mL of CMH reagent
Cal 4	0.1	1 mL of Cal3 + 1 mL of CMH reagent
Cal 5	0.05	1 mL of Cal4 + 1 mL of CMH reagent
Cal 6	0.025	1 mL of Cal5 + 1 mL of CMH reagent

TABLE 2 Quality Controls

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

NOTE 1—The source of the reagents is shown for information purposes only to aid laboratories initiating this procedure. Equivalent reagents from other suppliers can be used. Unless otherwise stated, reagents should be used within their stated validity period (for example, before the manufacturer's expiration date); processed reagents can have storage or use periods shorter than the source chemical or material.

8. Preparation of Standards and Controls

NOTE 2—Aseptic precautions are not needed, but contamination of reagents to be stored shall be avoided.

8.1 *Preparation of CMH Reagent*—The CMH reagent is supplied as a ready-to-use liquid.

8.2 *Preparation of Calibration Standards*—Hemoglobin standard is a commercially available reagent supplied as a solution with a fixed concentration. This stock is diluted in the CMH reagent to prepare calibration standards; mixing of diluted samples is conducted by vortexing; volumes can be adjusted based on the need (see Table 1).

8.3 *Preparation of Quality Controls*—These controls are prepared from an independent aliquot of the hemoglobin stock reagent; volumes can be adjusted based on the need (see Table 2).

8.4 *Preparation of Positive Controls*—Prepare 10 % solution (v/v) of Triton X-100 in water. Prepare aliquots for daily use and store at –20 °C ± 3 °C for up to 2 months.

8.5 *Preparation of Negative Control*—Use Ca^{2+}/Mg^{2+}-free DPBS as the negative control. Store the stock solution at 4 °C ± 3 °C.

8.6 *Preparation of Nanoparticulate Test Samples:*

8.6.1 For the initial dose, use the highest concentration of nanoparticles that is well dispersed in a physiologic solution. If the concentration in the end-use application is known, that can be used as the starting concentration. The material shall be well characterized under physiological conditions according to standard methods including those recommended in Practices F1877 and F1903. The nanoparticulate material for testing shall be in physiologic solution which is isotonic with a pH of 7.2 ± 2. This solution shall be defined and the particles shall be characterized in this solution. The number of particles/mL and mg/mL shall be indicated. Prior characterization shall be performed as appropriate to allow adequate data interpretation. For example, lot-to-lot variations in particle size and surface characteristics of the particles could result in different assay results. If the particles suspension is sterile, the method of sterilization shall be indicated. The nanoparticulate material and the buffer used for its storage/reconstitution shall be tested in the same assay. The assay requires at least 300 mL of test material and enough for diluting. The starting suspension shall be diluted in DPBS with serial one to five (1:5) dilutions at least three times to give four test samples in the assay.

8.6.2 Since some nanoparticulate materials can absorb at the designated 540-nm wavelength, it is suggested that a trial of the material in DPBS be tested at 540 nm. If absorption is evident, it is advisable to determine if high-speed centrifugation will pellet the particles and remove the interference. The maximum centrifugal force attainable using a microcentrifuge is typically 16 000 *g* to 22 000 *g*, corresponding to speeds upwards of 18 000 r/min. (See Note 3.) For this procedure, the maximum force available should be applied for 30 min. If successful, lower speeds can be tested if desired. When centrifugation is not applicable, an assay result obtained for a particle incubated with blood is adjusted by subtracting result obtained for the same particle in "minus blood" control (see 10.4).

NOTE 3—The relationship between centrifugation speed and the relative centrifugal force (RCF, measured in multiples of *gg*, the force of gravity at the Earth's surface) depends on the radius (r) of the rotor, where RCF = (r/min/1000) 2 × 1.12 × r (mm).

8.7 *Preparation of Blood Sample:*

8.7.1 Collect whole blood in tubes containing Li-heparin as the anticoagulant from at least three donors. The blood can be stored at 2 °C to 8 °C for up to 48 h. On the day of assay, prepare pooled blood by mixing equal proportions of blood from each donor. If microaggregates of blood are observed, filter the blood through a 40-µm blood filter.

8.7.2 Take a 2-mL to 3-mL aliquot of the pooled blood and centrifuge 15 min at 800 *g*.

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

9. Determination of PFH and TBH in Native Blood Sample

9.1 Add 200.0 µL of each calibration standard, quality control, and blank CMH reagent per well on 96-well plate. Use two wells for each calibrator and four wells for each QC and the blank so that test samples are bracketed by QCs; for example, a sequence such as (blank, Cal1, Cal6, QC1, QC2, QC3, test samples, blank, QC1, QC2, QC3).

9.2 Prepare the TBH sample by combining 20.0 µL of the pooled whole blood and 5.0 mL of CMH reagent. After 15 min, add 200.0 µL to each of six wells.

9.3 Add 100.0 µL of plasma (8.7.3) to six wells and add 100.0 µL of CMH reagent to each of these wells. Do not add CMH reagent to wells containing calibration standards and quality controls.

9.4 Cover the plate with the plate sealer and gently shake on a plate shaker (medium speed settings 2 to 3).

9.5 Read the absorbance at 540 nm to determine the hemoglobin concentration. Remember to use a dilution factor of 2 for the PFH sample and a dilution factor of 251 for TBH. If the calculated PFH concentration is below 1 mg/mL, proceed to the next step. *If it is above 1 mg/mL, the blood sample is not suitable for the procedure.*

10. Procedure with the Test Material

10.1 Dilute pooled whole blood with Ca^{2+}/Mg^{2+}-free DPBS to adjust TBH concentration to 10 mg/mL ± 1 mg/mL based on results from 9.5.

10.2 Add 100.0 µL of sample, reagent blank (PBS or other buffer used to dissolve nanoparticles), positive control solution, and negative control solution to microcentrifuge tubes. Prepare six tubes for each unknown sample, three tubes for the blank, two tubes for the positive control, and two tubes for the negative control.

10.3 Add 700.0 µL of Ca^{2+}/Mg^{2+}-free DPBS to each tube.

10.4 Add 100 µL of the whole blood prepared in 10.1 to each tube, except for three 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 (for example, absorbance at or close to 540 nm, reactivity with CMH reagent, and so forth).

10.5 Cover tubes and gently rotate to mix. At each 30-min interval, observe whether the sample of nanoparticles flocculate, disperse, sink, or float during testing.

NOTE 4—Vortexing can damage erythrocytes and shall be avoided.

10.6 Place the tubes in a water bath set at 37 °C ± 1 °C and incubate for 3 h ± 15 min mixing the sample by rotation every 30 min. Alternatively, tubes may be incubated on a tube rotator in an incubator set at 37 °C ± 1 °C.

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

10.8 Centrifuge the tubes for 15 min at 800 *g*.

10.9 When centrifugation is complete, examine the tubes and record any unusual appearance of the supernatant or pellet that can indicate additional damage to the red blood cells or the hemoglobin or adsorption of hemoglobin to the particles. See Fig. 1[6] and Section 13, Interferences.

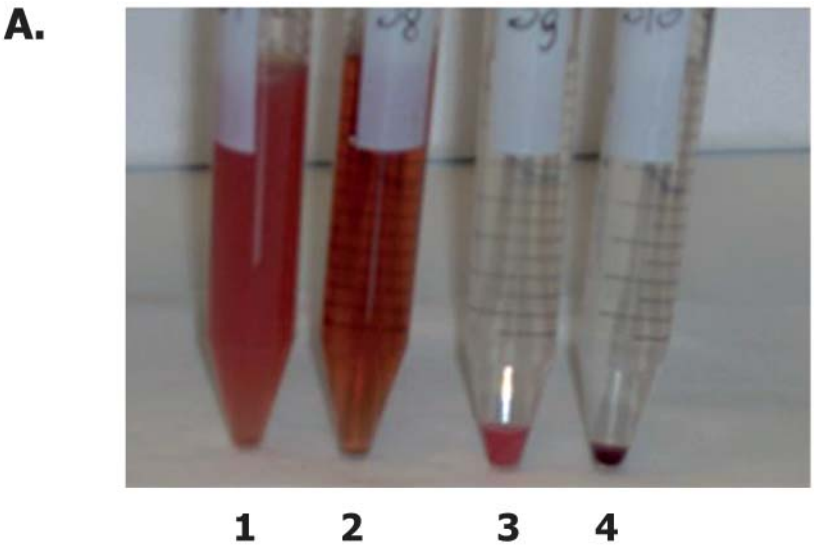
11. Determination of Hemolysis

11.1 Prepare fresh set of calibrators and quality controls as in 8.2 and 8.3.

11.2 To a fresh 96-well plate, add 200.0 µL of blank CMH, calibrators, quality controls, or diluted TBHd prepared by combining 400.0 µL of blood from 10.1 with 5.0 mL of CMH reagent. Fill two wells for each calibrator, four wells for each blank and each quality control, and six wells for the TBHd sample. As before, position all test samples bracketed between quality controls on the plate.

11.3 Add 100.0 µL per well of test samples, positive and negative controls, prepared in 10.9. Fill six wells for each test sample (two wells from each of three tubes prepared in 10.2) and four wells for each control (two wells from each of two tubes).

[6] DeSilva, B., Smith, W., Weiner, R., Kelley, M., Smolec, J., et al., "Recommendations for the Bioanalytical Method Validation of Ligand-Binding Assays to Support Pharmacokinetic Assessments of Macromolecules," *Pharmaceutical Research*, Vol 20, No. 11, November 2003, pp. 1885–1900.



(A.) In this example, polystyrene nanoparticles were hemolytic, but they adsorbed plasma free hemoglobin, which resulted in a false-negative assay result. This interference is detected by observation of the large, colored pellet at the bottom of the tube 3. (B.) In this example, fullerene derivative nanoparticles with absorbance at 540 nm resulted in a false-positive assay result. Subtracting OD of the nanoparticle sample without (w/o) blood from that of the sample with blood and re-evaluating the resultant OD against the assay standard curve generated final result (adjusted fullerene derivative with blood) for the test sample. This figure is reproduced with permission. See footnote 6.

FIG. 1 Example of Nanoparticle Interference with the Assay

11.4 Add 100.0 µL of CMH reagent to each well containing sample and controls. Do not add CMH reagent to wells containing calibration standards, quality controls, and TBHd that already contain CMH.

11.5 Cover the plate with plate sealer and gently shake on a plate shaker (medium speed settings 2 to 3).