



Designation: E 2524 – 08

## Standard Test Method for Analysis of Hemolytic Properties of Nanoparticles<sup>1</sup>

This standard is issued under the fixed designation E 2524; 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 ( $\epsilon$ ) 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 F 756 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 given in SI units are to be considered as the 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 and health practices and determine the applicability of regulatory limitations prior to use.*

### 2. Referenced Documents

#### 2.1 ASTM Standards:<sup>2</sup>

F 748 Practice for Selecting Generic Biological Test Methods for Materials and Devices

F 756 Practice for Assessment of Hemolytic Properties of Materials

F 1877 Practice for Characterization of Particles

F 1903 Practice for Testing For Biological Responses to Particles *in vitro*

#### 2.2 ISO Standard:

ISO 10993-4 Biological Evaluation of Medical Devices Part 4: Selection of Tests for Interactions with Blood<sup>3</sup>

### 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 *PEG*—polyethylene glycol

3.1.5 *PFH*—plasma-free hemoglobin

3.1.6 *QC*—quality controls

3.1.7 *TBH*—total blood hemoglobin

3.1.8 *TBHD*—blood sample diluted to 10 mg  $\pm$  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,<sup>4</sup> hemoglobin and its derivatives, such as sulhemoglobin, 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 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  $\mu$ L per test replicate.

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 F 748 and ISO 10993-4 to assess the biocompatibility of materials contacting blood in medical applications.

5.2 This test method is similar to Practice F 756 but modified to accommodate nanoparticulate materials.

### 6. Apparatus

6.1 Pipettes covering range from 0.05 to 10 mL.

6.2 Ninety-six well plates.

<sup>1</sup> This test method is under the jurisdiction of ASTM Committee E56 on Nanotechnology and is the direct responsibility of Subcommittee E56.02 on Characterization: Physical, Chemical, and Toxicological Properties.

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

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

<sup>4</sup> International Committee for Standardization in Haematology, *J. Clin. Pathol.*, Vol 31, 1978, pp. 139-143.



TABLE 1 Calibration Standards

| Level | Nominal Conc.,<br>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.,<br>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 $\mu$ L of QC1 + 800 $\mu$ L of CMH reagent   |
| QC 3  | 0.0625                  | 100 $\mu$ L of QC1 + 900 $\mu$ L of CMH reagent   |

6.3 Water bath set at  $37 \pm 1^\circ\text{C}$ .

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 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.<sup>5</sup> 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.

7.3 Hemoglobin standard.

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

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

7.6 Poly-L-lysine hydrobromide, MW 150 000 to 300 000.

7.7 PEG, average MW 8000.

7.8 Distilled water.

NOTE 1—The source of the reagents is shown for information purposes only to aid laboratories initiating this procedure. Equivalent reagents from other suppliers may be used.

## 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 Calibration Standards*—Volumes can be adjusted based on the need (see Table 1).

8.2 *Preparation of Quality Controls*—Volumes can be adjusted based on the need (see Table 2).

8.3 *Preparation of Positive Controls*—Dissolve poly-L-lysine hydrobromide powder to a final concentration of 1 % (10 mg/mL) in sterile distilled water. Prepare aliquots for daily use and store at  $-20 \pm 3^\circ\text{C}$  for up to 2 months. Alternatively, a 10 % solution of Triton-X 100 in water can be used as a positive control.

8.4 *Preparation of Negative Control*—PEG is supplied as 40 % stock solution in water. Use this solution as the negative control. Store the stock solution at  $4 \pm 3^\circ\text{C}$ .

### 8.5 Preparation of Nanoparticulate Test Samples:

8.5.1 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 may be used as the starting concentration. The material shall be well characterized under physiological conditions according to standard methods including those recommended in Practices F 1877 and F 1903. The nanoparticulate material for testing shall be in physiologic solution which is isotonic with a pH of  $7.2 \pm 2$ . 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.

8.5.2 Since some nanoparticulate materials may 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. 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).

### 8.6 Preparation of Blood Sample:

8.6.1 Collect whole blood in tubes containing Li-heparin as the anticoagulant from at least three donors. The blood can be stored at  $2$  to  $8^\circ\text{C}$  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- $\mu\text{m}$  blood filter.

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

8.6.3 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  $\mu\text{L}$  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

<sup>5</sup> *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. Pharmacopoeial Convention, Inc. (USPC), Rockville, MD.

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  $\mu\text{L}$  of the pooled whole blood and 5.0 mL of CMH reagent. After 15 min, add 200.0  $\mu\text{L}$  to each of six wells.

9.3 Add 100.0  $\mu\text{L}$  of plasma (8.6.3) to six wells and add 100.0  $\mu\text{L}$  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  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free DPBS to adjust TBH concentration to  $10 \pm 1$  mg/mL based on results from 9.5.

10.2 Add 100.0  $\mu\text{L}$  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  $\mu\text{L}$  of  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free DPBS to each tube.

10.4 Add 100  $\mu\text{L}$  of the whole blood prepared in 10.1 to each tube, except for three tubes of each test sample. In these tubes, add 100  $\mu\text{L}$  of  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -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 3—Vortexing may damage erythrocytes and shall be avoided.

10.6 Place the tubes in a water bath set at  $37 \pm 1^\circ\text{C}$  and incubate for  $3 \text{ h} \pm 15 \text{ min}$  mixing the sample by rotation every 30 min. Alternatively, tubes may be incubated on a tube rotator in an incubator set at  $37 \pm 1^\circ\text{C}$ .

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.

10.10 In Fig. 1, commercially available preparations of polystyrene nanoparticles with size 20 nm (Tube 1) and 50 nm (Tube 2) demonstrated hemolytic activity that can be observed by the color of the supernatant. Polystyrene nanoparticles with size 80 nm were also hemolytic; however, they adsorbed hemoglobin as can be determined by the pellet size and color (Tube 3). If the supernatant of this sample is used in the assay, the absorbance at 540 nm will demonstrate a negative result. Sample #4 is the negative control. No hemolytic activity was observed in the supernatant, and the intact red blood cells formed a tight dark red pellet on the bottom of the tube.

10.11 If the nanoparticles have an absorbance at or close to 540 nm, removal of these particles from the supernatant will be required before proceeding to the next step (see 8.5). These supernatants shall be transferred to fresh tubes and centrifuged 30 min at 40 000 rpm or speed determined in 8.5.

## 11. Determination of Hemolysis

11.1 Prepare fresh set of calibrators and quality controls as in 8.1 and 8.2.

11.2 To a fresh 96-well plate, add 200.0  $\mu\text{L}$  of blank CMH, calibrators, quality controls, or diluted TBHd prepared by combining 400.0  $\mu\text{L}$  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.

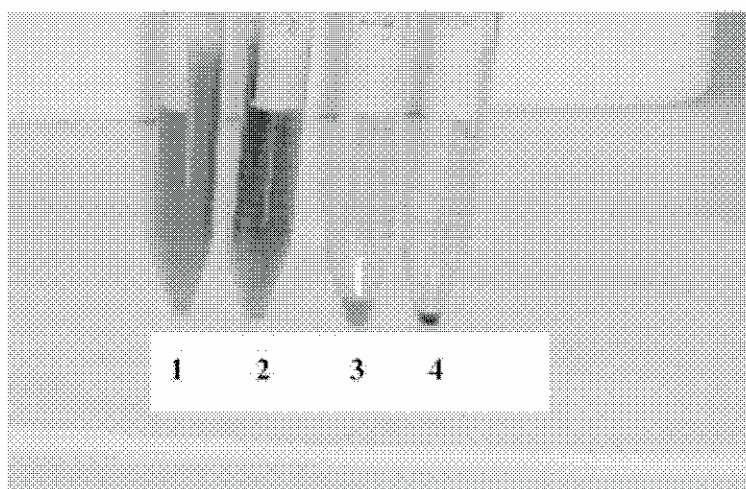


FIG. 1 Example Demonstrating the Importance of Recording Sample Appearance After Centrifugation to Avoid False Negative Results



11.3 Add 100.0  $\mu\text{L}$  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).

11.4 Add 100.0  $\mu\text{L}$  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).

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

## 12. Calculation

12.1 A four-parameter regression algorithm is used to build the calibration curve. The following parameters shall be calculated for each calibrator and quality control sample:

12.1.1 *Percent Difference from Theoretical:*

$$\text{PDFT} = \frac{(\text{Calculated Concentration} - \text{Theoretical Concentration})}{\text{Theoretical Concentration}} \times 100 \% \quad (1)$$

12.1.2 %CV shall be calculated for each blank, positive control, negative control, and unknown sample:

$$\text{Percent Coefficient of Variation: \%CV} = \text{SD}/\text{Mean} \times 100 \% \quad (2)$$

12.1.3 Percent hemolysis:

$$\% \text{ Hemolysis} = \text{PFH}_{\text{sample}}/\text{TBHd} \times 100 \% \quad (3)$$

## 13. Acceptance Criteria

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

13.2 The %CV for each positive control, negative control, and unknown sample shall be within 20 %.

13.3 If both replicates of positive control or negative control fail to meet the acceptance criterion described in 13.1, the run shall be repeated.

13.4 Within the acceptable run, if two of three replicates of unknown sample fail to meet acceptance criteria described in 13.1, this unknown sample shall be reanalyzed.

## 14. Report

14.1 Determine the % hemolysis caused by the test sample. A % hemolysis >5 % indicates that the test material will cause damage to red blood cells. Determine if there is a dose response curve.

## 15. Precision and Bias

15.1 Precision and bias have not been determined for this test method and will be determined within 5 years of publication of the standard. At this time there is no commercially available test article of sufficient quantity and reproducible quality to conduct intra- or interlaboratory comparisons of precision and bias.

## 16. Keywords

16.1 hemolysis; nanoparticulate; plasma; whole blood

## APPENDIX

### (Nonmandatory Information)

#### X1. RATIONALE

X1.1 This test method was modified from Practice F 756 to accommodate the use of nanoparticulate material. This test method was developed using human blood anticoagulated with heparin. Practice F 756 uses rabbit blood anticoagulated with citrate.



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- (2) DeSilva, B., Smith, W., Weiner, R., Kelley, M., Smolec, J., et al, "Recommendations for the Bioanalytical Method Validation of

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