Designation: E2524 – 08

StandardTest 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 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:*[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 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[3]

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 ± 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 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.

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.

[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.

Current edition approved Feb. 1, 2008. Published April 2008. DOI: 10.1520/E2524-08.

[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, *J. Clin. Pathol.* Vol 31, 1978, pp. 139-143.

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 to 10 mL.

6.2 Ninety-six well plates.

6.3 Water bath set at 37 ± 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 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.

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 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.

[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

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 ± 3°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 ± 3°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 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.

8.5.2 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°C for up to 48 h. On the day of assay, prepare pooled blood by mixing equal proportions of blood from each