



Designation: E3409 – 24

Standard Test Method for Analysis of Liposomal Drug Formulations Using Multidetector Asymmetrical-Flow Field-Flow Fractionation¹

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1. Scope

1.1 This test method describes a measurement procedure to reproducibly separate component size populations present within liposomal drug formulations and to characterize their associated size and size distribution. The method can also yield information on the shape and physical stability of the liposomes and is applicable to measurements in the presence of serum proteins. Fractions can be collected for off-line analysis using various techniques not specified in this test method.

1.2 This test method applies to uni-lamellar and multi-lamellar liposomes that are designed for drug delivery and which are dispersed in a native solution that is aqueous in nature. The method is generally applicable over a particle size range (radius) of approximately 10 nm to 250 nm, and for injected lipid mass from 20 μg to 200 μg .

1.3 This test method is based on the multi-detector asymmetrical-flow field-flow fractionation (MD-AF4) technique as configured on a typical commercial instrument platform with online detectors such as multi-angle (static) light scattering (MALS), dynamic light scattering (DLS), ultraviolet-visible (UV-Vis) absorbance, and differential refractive index (dRI) (1).²

1.4 This method does not address liposome composition. Refer to Test Methods E3297, E3323, or E3324 for lipid quantification.

1.5 *Units*—The values stated in SI units are to be regarded as standard. Where appropriate, cgs units are given in addition to SI.

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 This test method is under the jurisdiction of ASTM Committee E56 on Nanotechnology and is the direct responsibility of Subcommittee E56.02 on Physical and Chemical Characterization.

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² The boldface numbers in parentheses refer to a list of references at the end of this standard.

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:³

D1193 Specification for Reagent Water

E1617 Practice for Reporting Particle Size Characterization Data

E3144 Guide for Reporting the Physical and Chemical Characteristics of Nano-Objects

E3206 Guide for Reporting the Physical and Chemical Characteristics of a Collection of Nano-Objects

E3247 Test Method for Measuring the Size of Nanoparticles in Aqueous Media Using Dynamic Light Scattering

E3297 Test Method for Lipid Quantitation in Liposomal Formulations Using High Performance Liquid Chromatography (HPLC) with a Charged Aerosol Detector (CAD)

E3323 Test Method for Lipid Quantitation in Liposomal Formulations Using High Performance Liquid Chromatography (HPLC) with an Evaporative Light-Scattering Detector (ELSD)

E3324 Test Method for Lipid Quantitation in Liposomal Formulations Using Ultra-High-Performance Liquid Chromatography (UHPLC) with Triple Quadrupole Mass Spectrometry (TQMS)

2.2 ISO Standards:⁴

ISO/TS 21362 Nanotechnologies—Analysis of Nano-Objects Using Asymmetrical-Flow and Centrifugal Field-Flow Fractionation

ISO 22412 Particle Size Analysis—Dynamic Light Scattering (DLS)

³ 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.

⁴ Available from International Organization for Standardization (ISO), ISO Central Secretariat, Chemin de Blandonnet 8, CP 401, 1214 Vernier, Geneva, Switzerland, <http://www.iso.org>.

2.3 *Code of Federal Regulations*:⁵

21 CFR § 211.194(a)(2) FDA Current Good Manufacturing Practice for Finished Pharmaceuticals

3. Terminology

3.1 Definitions:

3.1.1 *accumulation wall*, *n*—surface of a field-flow fractionation channel toward which sample components are driven by the applied field acting perpendicular to the channel flow.

ISO/TS 21362

3.1.1.1 *Discussion*—In asymmetrical-flow field-flow fractionation, the accumulation wall is flat and consists of a replaceable semipermeable membrane on a porous frit substrate.

3.1.2 *asymmetrical-flow field-flow fractionation (AF4)*, *n*—separation technique that uses a cross flow field applied perpendicular to the channel flow to achieve separation based principally on analyte diffusion coefficient or size. **Adapted from ISO/TS 21362**

3.1.3 *band broadening*, *v*—overall dispersion or widening of an analyte band as the analyte passes through a separation system.

ISO/TS 21362

3.1.4 *channel*, *n*—in field-flow fractionation, a thin ribbon-like chamber with a parabolic flow profile required for separation under the influence of a field applied perpendicular to the channel flow.

ISO/TS 21362

3.1.5 *channel flow*, *n*—in field-flow fractionation, the total parabolic laminar-flow of eluent or mobile phase through the channel.

Adapted from ISO/TS 21362

3.1.5.1 *Discussion*—For purposes of this test method, channel flow and detector flow are equivalent. If the operator uses a post-fractionation split-flow device to divert part of the channel flow to waste (for example, to concentrate the analyte), then the detector flow will be less than the channel flow.

3.1.6 *channel spacer*, *n*—in asymmetrical-flow field-flow fractionation, a thin plastic film with a cut-out that defines the nominal thickness and lateral dimensions of a channel.

Adapted from ISO/TS 21362

3.1.6.1 *Discussion*—The geometry of the channel is typically defined by a trapezoid shape with breadth typically ca. 20 mm to 25 mm, length typically ca. 100 mm to 300 mm and height typically between 190 μm and 500 μm . The nominal channel height and lateral dimensions can also be defined using fixed-height channels that do not require a spacer.

3.1.7 *channel thickness (height)*, *w*, *n*—in field-flow fractionation, the nominal vertical separation between the depletion wall and the accumulation wall.

3.1.7.1 *Discussion*—The effective thickness can vary depending on the membrane used and the degree of compression of the membrane as well as other factors.

3.1.8 *cross flow*, *n*—in asymmetrical-flow field-flow fractionation, the flow field applied perpendicular to the channel flow to achieve separation of analytes. **ISO/TS 21362**

3.1.9 *detector flow*, *n*—in field-flow fractionation, that portion of mobile phase that exits the channel and enters the detectors.

Adapted from ISO/TS 21362

3.1.9.1 *Discussion*—While typically detector flow and channel flow are identical, in some configurations channel flow can be split before entering the detectors, for example, to concentrate the analyte by purging a portion of the analyte-free mobile phase.

3.1.10 *elution*, *n*—in field-flow fractionation, a process by which analytes in the mobile phase, or eluent, are transported through, and exit from, the fractionation channel.

ISO/TS 21362

3.1.11 *elution time*, *t*, *n*—in field-flow fractionation, the elapsed time beginning with the initiation of elution following sample injection and excluding pre-elution steps such as focusing, relaxation or transitions.

3.1.11.1 *Discussion*—Retention and elution share an equivalent timeline and may be used interchangeably. However, retention is principally used in reference to specific retained species (for example, an analyte) eluting more slowly than the unretained tracer (that is, the void peak) due to their specific properties and interaction with the applied field. A graphical representation of a fractogram generally shows elution time or retention time on the (horizontal) x-axis, after the time associated with pre-elution steps has been subtracted.

3.1.12 *field-flow fractionation*, *n*—separation technique where a field is applied to a liquid passing along a narrow channel to induce separation of analytes present in the liquid, dependent on their differing mobility under the force exerted by the field.

Adapted from ISO/TS 21362

3.1.13 *focusing*, *v*—in asymmetrical-flow field-flow fractionation, a process by which, during and after sample injection, a counter-balanced flow entering from opposite directions in the channel is applied to focus the sample components into a thin band close to the inlet port and near the accumulation wall.

Adapted from ISO/TS 21362

3.1.13.1 *Discussion*—While conventional channels require a focusing step, there are channel designs, such as the frit-inlet channel, that do not require focusing. Focusing is also associated with the process of sample relaxation.

3.1.14 *fractogram*, *n*—in field-flow fractionation, a two-dimensional graphical representation of data derived from an experiment, typically with one or more detector signals on the vertical y-axis and time on the horizontal x-axis.

Adapted from ISO/TS 21362

3.1.15 *hydrodynamic radius*, R_h , *n*—the sphere-equivalent ensemble average radius that reflects the central tendency of the underlying population of particles as determined by dynamic light scattering.

Adapted from Test Method E3247

3.1.15.1 *Discussion*—The hydrodynamic radius can be obtained from different computational methods, including, for instance, the cumulants method combined with the Stokes-Einstein equation. Note that when obtained from cumulants the average hydrodynamic radius is often referred to in the literature as the z-average (z-avg) size, a legacy term. This measurand can be scattering-angle-dependent and can be influenced by other factors besides size.

⁵ Available from U.S. Government Publishing Office (GPO), 732 N. Capitol St., NW, Washington, DC 20401, <http://www.gpo.gov>.

3.1.16 *injection flow, n—in field-flow fractionation*, flow that drives the sample out of the injection loop and into the fractionation channel. **ISO/TS 21362**

3.1.17 *liposomal formulation, n—product designed to assist in the delivery of an active pharmaceutical ingredient either encapsulated or intercalated in the liposome.*

3.1.17.1 *Discussion*—Water-soluble drugs are contained in the aqueous compartment and hydrophobic drugs are contained within the lipid bilayer. Additional molecular species are incorporated into the bilayer to control functionality (for example, distearoyl phosphatidylethanolamine-PEG2000-amine is commonly used to confer colloidal stability and minimize non-specific interactions). The finished drug product might also contain inactive ingredients (excipients) in addition to the lipids and active pharmaceutical ingredient(s).

3.1.18 *liposome, n—synthetic vesicle composed of one or more bilayers formed by amphipathic molecules such as phospholipids that enclose one or more aqueous compartments and hydrophobic regions.*

3.1.19 *mobile phase (eluent), n—liquid phase used to achieve transport and separation of analytes in field-flow fractionation or chromatography.*

3.1.20 *recovery, n—in field-flow fractionation*, ratio of the analyte mass eluted during fractionation to the initial injected mass, expressed as a percentage. **ISO/TS 21362**

3.1.20.1 *Discussion*—In practice, recovery is determined using an appropriate online mass concentration detector by comparing the area under the mass concentration trace obtained with and without application of the applied field and focusing. Recovery is considered acceptable if at or above 70 % according to ISO/TS 21362; higher recoveries are anticipated for high quality (for example, clinical) liposomal drug formulations. In this test method, recovery includes all mass eluted during the elution program (post void peak), including any residual material released after cessation of cross flow (report this value as “total recovery”). The user may also choose to report a recovery value that excludes residual material (that is, material eluting after cross flow is removed). In this case, the recovery value should be reported as “field-on recovery.”

3.1.21 *relaxation, v—in field-flow fractionation*, process by which the sample components assume their equilibrium state with respect to the opposing forces of diffusion and the applied field before elution is initiated. **ISO/TS 21362**

3.1.22 *residual peak, n—in field-flow fractionation*, a peak appearing in the fractogram after the applied field is removed (field-off) and which corresponds to unfractionated material retained on the accumulation wall during application of the applied field.

3.1.22.1 *Discussion*—This “peak” often contains particles or fragments that are too large to migrate under the applied field or which for some other reason strongly interact with the membrane or channel surface. The peak is frequently ill-defined (that is, atypical shape).

3.1.23 *retention ratio, R, n—in field-flow fractionation*, the ratio of the velocity of the analyte to the average velocity of the fluid in the channel.

3.1.23.1 *Discussion*—Typically expressed as the ratio of the void peak elution time to the analyte peak retention time. For efficient separation, R should be less than 0.2.

3.1.24 *retention time, t_R , n—in field-flow fractionation*, the time between initiation of fractionation and detection of an analyte peak defined at its maximum signal intensity. **ISO/TS 21362**

3.1.24.1 *Discussion*—Retention time for an analyte is generally determined from the mass concentration detector signal at an elution time associated with the fractionated analyte peak. Retention time and elution time share the same effective timeline. In principle, retention time can be assigned based on any signal that tracks the elution of the analyte. Note that retention time for many colloidal particles, including liposomes, can differ between mass concentration and light scattering detectors due to differences in detector sensitivity toward analyte size.

3.1.25 *root mean square radius (radius of gyration), R_g , n—in multi-angle static light scattering*, the root mean square distance of a scattering object’s component parts from its center of mass.

3.1.25.1 *Discussion*—In a light scattering measurement, object refers to a microscopic particle dispersed in a liquid that scatters light with a differential angular distribution of intensity.

3.1.26 *selectivity, S, n—in field-flow fractionation*, a measure of fractionation power related to the change in retention time relative to the corresponding change in particle size.

3.1.26.1 *Discussion*—Selectivity can be determined experimentally from the slope of $\log t_R$ versus $\log 2r$, where r is radius measured by MALS or DLS. These measurements are typically performed under conditions similar to those used for sample analysis, but with materials of known size (for example, PSL spheres). For AF4, S can exceed unity, where the higher the value of S , the better the resolving power.

$$S = \left| \frac{d \log t_R}{d \log 2r} \right| \quad (1)$$

3.1.27 *void peak, n—in field-flow fractionation*, a peak appearing early in the fractogram that corresponds to unretained, typically small sample components that are not in equilibrium with the separation field. **ISO/TS 21362**

3.1.27.1 *Discussion*—The void peak, typically very small and narrow, is used to define the void time t_0 . The void peak is always present as an artifact of the hardware change from focus mode to elution mode, and elutes at the average velocity of the channel flow. An uncharacteristically large void peak indicates substantial unretained material is present, suggesting the elution conditions might not be acceptable for that sample; further method development might be necessary to resolve this issue, or the sample might not be appropriate for AF4 analysis.

3.1.28 *void time, t_0 , n—in field-flow fractionation*, the elution time associated with the maximum of the void peak.

3.2 *Acronyms and Symbols:*

3.2.1 *AF4*—asymmetrical-flow field-flow fractionation

3.2.2 *BSA*—bovine serum albumin

3.2.3 *CF*—channel flow—flow exiting the channel; equivalent to *DF* in this method

3.2.4 *DAD*—diode array detector (UV-Vis absorbance)

3.2.5 *DF*—detector flow; equivalent to *CF* in this test method

3.2.6 *dRI*—differential refractometer or differential refractive index

3.2.7 *DLS*—dynamic light scattering

3.2.8 *DPBS*—Dulbecco's phosphate buffered saline

3.2.9 *FF*—focus flow

3.2.10 *FWHM*—full width at half maximum

3.2.11 *IF*—injection flow; sample injection

3.2.12 *MALS*—multi-angle (static) light scattering

3.2.13 *MD*—multi-detector

3.2.14 *MWCO*—molecular weight cut-off

3.2.15 *PBS*—phosphate buffered saline

3.2.16 *PES*—polyethersulfone

3.2.17 *PSL*—polystyrene latex (sphere, bead)

3.2.18 *RC*—regenerated cellulose

3.2.19 *R*—retention ratio

3.2.20 R_g —radius of gyration (root mean square radius)

3.2.21 R_h —sphere-equivalent hydrodynamic radius

3.2.22 $R(\%)$ —analyte recovery

3.2.23 *S*—selectivity

3.2.24 t_o —void time

3.2.25 t_R —retention time

3.2.26 *UV-Vis*—ultraviolet-visible (wavelength range)

3.2.27 *w*—channel thickness (nominal as defined by a removable spacer or as defined by a fixed-height channel with no spacer)

3.2.28 *XF*—cross flow

4. Summary of Test Method

4.1 Liposomal drug products are diluted into filtered PBS to achieve a total lipid concentration of 1 mg/mL. The diluted test specimen is injected into the MD-AF4 system using PBS as the mobile phase. A total injected lipid mass of 25 µg to 50 µg is targeted.

4.2 Test specimens are analyzed with and without applied cross flow and focusing in order to estimate analyte recovery.

4.3 Particle size (root mean square radius and, optionally, hydrodynamic radius) is measured online during elution under cross flow following a standardized procedure (1) that is generally compliant with ISO/TS 21362.

4.4 Prior to sample analysis, instrument system performance is verified using BSA in PBS mobile phase. Traceable PSL spheres (for the applicable size range) can be used with an appropriate mobile phase (see Appendix X3) to verify that the

instrument settings (for example, MALS angles, DLS angle, detector flow rate, cell configuration) allow for accurate analysis of particles in the size range of interest for application of the test method.

4.5 Calibration/normalization of the MALS detector is performed periodically according to manufacturer instructions. Mass concentration detector linearity is determined over the mass concentration range of interest according to manufacturer instructions on a periodic basis to ensure meaningful recovery determination. This test method presumes that primary instrument qualification has been performed by the manufacturer or according to their recommendations.

5. Significance and Use

5.1 Liposomal formulations for the treatment of cancer and other diseases are the most common form of nanotechnology-enabled drug products submitted for market approval and in clinical application at the present time. The accurate characterization of their physical-chemical properties is critical to support the development and assessment of such products (2). In particular, size, size distribution, shape, and physical stability are key properties (among others) that have been widely identified as critical quality attributes (CQAs) for liposomal drug products.

5.1.1 Asymmetrical-flow field-flow fractionation (AF4) is a chromatographic-like technique that uses hydrodynamic forces to gently separate analytes into their component populations according to size and diffusivity (3 and 4). The fractionated sample then passes through one or more online detectors chosen specifically for the application requirements. The combination of low-shear separation, tolerance for complex matrices, and exceptionally broad size range make AF4 a technique of choice for application to nanotechnology-enabled drug products such as liposomes (13, 5-9).

5.1.2 Multi-detector AF4 is suitable for research and development, manufacturing quality control, product stability/shelf-life testing and regulatory assessments.

5.1.3 There are multiple assumptions inherent in the application of MD-AF4, including the appropriateness of models used to interpret online light scattering data and the compatibility of the analyte and matrix with the membrane that forms the surface of the accumulation wall in the fractionation channel. Other assumptions are detector or analyte specific.

5.2 Chemical components of the mobile phase must not induce agglomeration of liposomes or otherwise significantly alter their physical properties.

5.3 Discretion should be used in the interpretation of size data obtained by different scattering detectors using different scattering models and modes of analysis.

5.4 The Current Good Manufacturing Practice for Finished Pharmaceuticals (see 21 CFR 211.194(a)(2)) and the ICH Harmonized Tripartite Guideline on Validation of Analytical Procedures Q2(R1) (10), state that the suitability of all test methods shall be verified under actual conditions of use.

5.5 MD-AF4 can be compared with batch mode dynamic light scattering (DLS) for the determination of liposome mean size and size distribution, where MD-AF4 provides deconvolution of complex mixtures yielding a more accurate assessment of the populations present. Batch mode DLS (see, for instance, Test Method [E3247](#)) provides a rapid, low-cost approach that can be used to screen materials prior to analysis by MD-AF4. The latter requires substantially more analyst time and effort along with appropriate training and expertise. Qualitatively, MD-AF4 offers greater insight into the physical complexity of a sample via the fractionation process in combination with multiple detectors.

6. Interferences

6.1 Most common sources of interference that can adversely impact batch measurements of liposomes using light scattering-based methods are mitigated by the fractionation process in AF4, which yields data from nearly monodisperse population intervals.

6.2 Sample matrix components (for example, tonicity agents, surfactants or other molecular species) can interfere with the analysis of analyte size if not separated from the analyte with sufficient resolution. This is generally not an issue for liposomes due to their larger size compared with common sample matrix components.

6.3 Sample matrix components can impact the calculated recovery, as sufficiently small species can pass through the accumulation wall membrane during focusing and field-on elution. This loss could reduce the mass concentration detector signal and thereby reduce the apparent recovery. This effect is significant only when the mass concentration detector is sensitive to these species (that is, they absorb strongly at 280 nm in the UV detector). This potential interference may be evaluated offline using centrifugal filtration or dialysis to separate the liposomal analyte from the interfering matrix components. The filtrate or dialysate can then be tested for absorbance at the operational UV wavelength. When these components are instead retained by the membrane, it may not be possible to differentiate their contribution to recovery determination.

6.4 A common interference for the application of this test method is analyte-membrane interaction in the fractionation channel. Such interactions can lead to unacceptably low recovery and biased results.

6.4.1 Memory effects can interfere with subsequent analyses when material is carried over from previous elution runs due principally to adherence on the accumulation wall membrane. Other surfaces in the fractionation channel, though less likely, can potentially contribute to this effect.

6.5 Scattering at the UV wavelength used to measure analyte mass can potentially interfere with the accurate determination of analyte recovery. Therefore, confirmation of the linearity of UV absorbance at 280 nm over the mass range of interest is recommended. Confirmation that scattering is not a significant interference can also be accomplished using a second independent mass concentration detector such as dRI.

6.6 This test method is designed to maintain physiological pH and osmolarity during analysis using PBS as the principal medium. Differences in the osmotic concentration or tonicity of the liposome drug product and the medium or mobile phase into which the product is diluted can potentially cause osmotic stress-induced changes to liposome size (shrinkage or swelling) and other structural transformations. Such effects are more likely to occur in low electrolyte media and increase in likelihood with increasing liposome size. If significant structural changes due to osmotic stress are suspected, the analyst may wish to consider a different methodology or evaluate modifications of the mobile phase that allow osmotic matching ([Note 1](#)). Any significant deviations from the proscribed method must be fit for purpose and clearly stated in the analysis report.

NOTE 1—To test for the presence of significant osmotic stress-induced physical changes, the analyst can evaluate the test material using, for example, a serial dilution of PBS.

7. Apparatus

7.1 See [Annex A1](#) for a detailed description of the MD-AF4 instrumentation required for this test method. See [Appendix X4](#) for schematic drawings representing three basic channel/flow designs in current or potential commercial use.

7.2 *AF4 Trapezoidal Channel* with a nominal channel thickness of 350 μm (either fixed-height or using a replaceable plastic spacer) and a nominal channel length between approximately 140 mm and 300 mm.

7.2.1 If a fixed-height channel with a different nominal height is used, it is the user's responsibility to verify it is fit for purpose and to report this change.

7.3 *Mechanical Pipettes and Disposable Pipette Tips*, ranging from 2 μL to 10 mL.

7.4 *Solvent Reservoir Bottles*, 1 L or larger.

7.5 *LC-MS Grade Autosampler Glass Vials (2 mL)* with polytetrafluoroethylene silicone septa-fitted caps (if using an autosampler in conjunction with the MD-AF4 system—these are unnecessary if a manual sample injector is used).

7.6 *Calibrated pH Meter* with pH electrode appropriate for aqueous solutions and suspensions.

8. Reagents and Materials

8.1 Unless otherwise stated, references to water shall be understood to mean reagent grade (ultrapure) water⁶ ($\geq 18.2 \text{ M}\Omega \text{ cm}$) conforming to Type I of Specification [D1193](#) and passed through a $\leq 0.22 \mu\text{m}$ pore size filter. Commercially available LC-MS grade water meets this specification for the purpose of this test method.

8.2 Reagent-grade chemicals shall be used in all tests. Unless otherwise indicated, it is required that all reagents

⁶ *ACS Reagent Chemicals, Specifications and Procedures for Reagents and Standard-Grade Reference Materials*, 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.

conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society,⁶ where such specifications are available. Other grades may be substituted, if necessary, provided it is first ascertained that the reagent is of sufficiently high purity to be fit for purpose.

8.3 Mobile Phase—The primary mobile phase or eluent for this test method is phosphate buffered saline (PBS), which nominally contains 8 g/L sodium chloride (NaCl), 0.2 g/L potassium chloride (KCl), 1.15 g/L sodium phosphate (Na_2HPO_4) and 0.2 g/L potassium phosphate monobasic (KH_2PO_4), with pH nominally 7.2 to 7.4. PBS was chosen because it provides physiological pH buffering and isotonic dilution of liposome drug formulations. All mobile phase solutions shall be filtered at $\leq 0.22 \mu\text{m}$ pore size prior to use.

8.3.1 Commercially available sterile PBS buffer formulations (without calcium, magnesium, glucose, pyruvate, and phenol red) can vary in their specific chemical composition, pH and designation (for example, Dulbecco's PBS or DPBS). The test method described here was evaluated using four commercial PBS products with similar compositions and pH values ranging from 7.2 to 7.4. Results indicate that all tested PBS products yield statistically identical results for liposome formulations based on sample recovery, separation efficiency and measured size (1). We conclude that the specific PBS product formulation used is not critical to the outcome of this test method. Any reagent grade, sterile PBS formulation without calcium, magnesium, glucose, pyruvate and phenol red, and with a pH from 7.2 to 7.4, is considered acceptable and fit for purpose.

8.3.2 Sterile isotonic saline (sodium chloride, 154 mmol/L or 9 g/L—commonly labelled as 0.9 % w/v) was evaluated as a secondary buffer and can be used in place of PBS in this test method and will yield similar results. The user should note that the pH of saline is slightly acidic (pH 6) and is not buffered.

8.4 *Quality Controls to Evaluate Instrument Performance:*

8.4.1 Bovine serum albumin (BSA) lyophilized powder (≥ 99 % purity, Fraction V) or ampouled BSA concentrate. BSA is utilized as a Rayleigh scatterer for normalization of MALS sensors (relative to 90° scattering angle primary calibration). BSA is also used as a routine verification material for overall instrument performance (flow rates, pressure, membrane condition, detector operation, etc.) and to detect changes in performance over time. Refer to [Appendix X2](#) for a general procedure to prepare BSA stock suspension.

8.4.2 Commercially available spherical polystyrene latex (PSL) size standards, with nominal diameters of, for example, 60 nm, 125 nm, 200 nm, and 350 nm, can be used to verify instrument performance with respect to size measurements (MALS and DLS) and selectivity (fractionation). Depending on the size of the liposome analyte, other nominal size PSLs can be chosen to bracket the analyte size. Only the PSL monomer peak is of interest in this context; dimers or other oligomers will elute later in time and can be ignored. Refer to [Appendix X3](#) for general procedures to prepare PSL samples for analysis.

8.4.2.1 Other (non-polystyrene) polymer latices may be used if fit for purpose. Principally, these particles should be

uniformly spherical, monomodal and with a very narrow size distribution (nearly monodisperse with polydispersity index less than 0.1).

8.5 Hydrophilic polyethersulfone (PES) syringe filters with $0.2 \mu\text{m}$ or $0.1 \mu\text{m}$ pore diameter and sterile plastic syringes are recommended to filter water or mobile phase prior to use. PES filter units (150 mL or larger) with a vacuum connector can be used to conveniently and quickly filter larger volumes of water, buffer or mobile phase. A different filter material can be substituted if verified to be fit for use.

8.6 Precut trapezoidal 10 kDa MWCO regenerated cellulose (RC) semipermeable membranes for use in the fractionation channel. Precut membranes are obtained from the instrument manufacturer to fit their standardized channels.

8.7 Plastic spacer ($350 \mu\text{m}$ nominal thickness) compatible with the specific AF4 channel that is used to implement this test method (for example, polyethylene terephthalate “Mylar” or other manufacturer recommended material is acceptable). If a fixed-height channel of the same nominal thickness (height) is used, a spacer is not needed.

8.8 In addition to those listed above, additional materials or chemicals might be required by the instrument manufacturer for the proper operation, maintenance and primary MALS calibration of specific instrument platforms. Follow manufacturer instructions where appropriate.

9. Hazards

9.1 Appropriate protective measures and disposal procedures are required while handling liposome drug formulations that contain hazardous or toxic components (for example, encapsulated chemotherapeutic drugs); refer to product safety information where available.

10. Mobile Phase Preparation

10.1 Rinse the solvent reservoir bottles at least three (3) times with sterile ultrapure water filtered using $\leq 0.22 \mu\text{m}$ pore size PES filter. The reservoir bottles should be clean and should be used exclusively for media containing water and simple salts.

10.2 Use commercially available (or in-lab prepared), sterile PBS or (optionally) isotonic saline for the mobile phase. Dilute commercial concentrates (for example, 10x PBS) using ultrapure filtered water. For preparation of in-lab mobile phase, refer to [Appendix X1](#) for details.

10.2.1 Measure and record the pH of the mobile phase.

10.3 Filter the mobile phase using $\leq 0.22 \mu\text{m}$ pore size PES filter. Then rinse the solvent reservoir bottles three (3) times with filtered mobile phase, before filling to nominal capacity.

10.4 Continuous circulation of mobile phase through an in-line filter (typical with commercial isocratic pumps) maintains sterile conditions throughout the system. Chemical disinfectants, such as sodium azide, are not recommended and have not been evaluated with the test method described here. Their inclusion requires verification by the user. When instrumentation is not being used for analysis, the system should be