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Orthogonal and complementary measurements of properties of drug products containing nanomaterials

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ABSTRACT

Quality control of pharmaceutical and biopharmaceutical products, and verification of their safety and efficacy, depends on reliable measurements of critical quality attributes (CQAs). The task becomes particularly challenging for drug products and vaccines containing nanomaterials, where multiple complex CQAs must be identified and monitored. To reduce (i) the risk of measurement bias and (ii) the uncertainty in decision-making during product development, the combination of orthogonal and complementary analytical techniques are generally recommended by regulators. However, despite frequent reference to "orthogonal" and "complementary" in guidance documents, neither term is clearly defined. How does one determine if two analytical methods are orthogonal or complementary to one another? Definitions are needed to design a robust characterization strategy aligned to regulatory needs. Definitions for "orthogonal" and "complementary" are proposed that are compatible with existing metrological terminology and are applicable to complex measurement problems. Orthogonal methods target the quantitative evaluation of the true value of a product attribute to address unknown bias or interference. Complementary measurements include a broader scope of methods that reinforce each other to support a common decision. Examples of the application of these terms are presented, with a focus on measurement of physical properties of nano-enabled drug products, including liposomes and polymeric nanoparticles for cancer treatment, lipid-based nanoparticles (LNPs) and virus-like particles for nucleic acid delivery. The proposed framework represents a first step in advancing the assessment of the orthogonality and complementarity of two measurements and it can potentially serve as the basis for a future international standard. This framework may help product developers to implement more efficient product characterization strategies, accelerate the introduction of novel medicines to the clinic and be applicable to other therapeutics beyond nanomaterial-containing pharmaceuticals.

Abbreviations: AF4, asymmetric flow field flow fractionation; API, active pharmaceutical ingredient; AUC, analytical ultracentrifugation; CF3, centrifugal field flow fractionation; CQAs, critical quality attributes; CyA, cyclosporin A; DLS, dynamic light scattering; FDA, Food and Drug Administration; EUNCL, European Nanomedicine Characterization Laboratory; GUM, Guide to the Expression of Uncertainty in Measurement; HPLC-IC, ion chromatography coupled to high performance liquid chromatography; ICP-MS, inductively coupled plasma mass spectrometry; ICP-OES, inductively coupled plasma - optical emission spectrometry; LD, Laser diffraction; LNPs, lipid-based nanoparticles; mAb, monoclonal antibody; MALS, multiangle light scattering; NIST, National Institute of Standards and Technology; NTA, nanoparticle tracking analysis; PSD, particle size distribution; Rg, geometric radius or radius of gyration; Rh, hydrodynamic radius; SEC, size exclusion chromatography; SLS, static light scattering; SOP, standard operating procedure; sp-ICP-MS, single particle-ICP-MS; TEM, transmission electron microscopy; VLPs, virus like particles..

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1. Introduction

Pharmaceutical quality underpins the safety and efficacy of the medicines that patients receive and quality control depends on reliable measurement of critical quality attributes (CQAs). The issue is amplified in the case of drug products containing nanomaterials, where multiple properties need to be measured during drug development for quality control [1] (Fig. S1 & S2). These include five additional aspects related to the presence of nanomaterials in the formulation (i) *the physical properties* of the particles, including their size/size distribution, morphology, and particle concentration (ii) *the chemical properties* of the drug substance and of the drug product (particle and encapsulated active pharmaceutical ingredient (API)) including the chemical composition and the purity of their components, (iii) *the spatially resolved structure* of the particles, where crystallinity plays an important role, (iv) *the particle surface coating*, surface charge and the coating interactions with the biological environment surrounding the particle surface (particle-proteins interactions) and finally (v) the *drug (API) payload* and drug *release* from the nanoparticle carrier. For each attribute, a variety of different characterization methods may be available.

Particle size distribution (PSD) is one of the most commonly measured attributes of drug products containing nanomaterials. There are many methods for measuring PSD which makes it a useful example for applying the concepts of orthogonality and complementarity. PSD can be difficult to assess and carries a risk of measurement bias. Measuring the size of a spherical nanoparticle would mean quantifying its physical diameter. In the case of nano-objects, size cannot be measured directly by visualisation with a classical optical microscope. There are many available techniques (e.g., dynamic light scattering (DLS), laser diffraction, nanoparticle tracking analysis (NTA), or electron microscopy (EM)) to perform the task. Each measurements depends on a different physical principle giving a different "type" of size, each providing a measured value for the same measurand. Measured values from different techniques on the same sample may vary, complicating the assessment of product quality.

It is often recommended to use a combination of *orthogonal* and *complementary* methods, to increase the likelihood that the critical properties of a complex product are characterized reliably. For example, complementary methods have been recommended in the characterization of drug products containing nanomaterials to address technique-related differences in measurements [2], while orthogonal methods are useful in other instances [3,4]. According to the draft ICH Q2(R2) guideline on Validation of Analytical Procedures, an orthogonal procedure comparison is when the results of an analytical method "are compared with those of a second well-characterized procedure that ideally applies a different measurement principle (independent procedure)." [5]. Similarly, for a monoclonal antibody (mAb) standard reference material, there is emphasis on using multiple *orthogonal techniques* to measure the CQAs of the mAb, including size and aggregation propensity, to confirm the validity of the results [6,7]. A PubMed search of titles containing "characterization" and "orthogonal" yields 370 publications [8]. An example is the review published by the European Nanomedicine Characterization Lab (EUNCL) and the National Cancer Institute - Nanotechnology Characterization Laboratory (NCI-NCL) that advocates the use of *orthogonal measurements* for PSD of drugs containing nanomaterials [9].

In the examples above, the terms "orthogonal" and "complementary" do not have clear definitions within the context of medical product characterization. Operationally, what the authors of the current work and the scientific community seem to propose, is that using different methods should help to obtain more reliable data than a single method. The combination of different sources of information that corroborate and reinforce each other yields results that are more informative and less biased. At the same time, only the necessary and appropriate methods should be used to avoid excessive cost and time in bringing medicines to the market.

In this perspective, definitions are proposed for "orthogonal" and "complementary" measurements in the context of medical product characterization. A framework for using the definitions is provided with examples where different measurements are compared to determine whether they are orthogonal or complementary. This perspective represents a first step in achieving consensus definitions for these terms within the community and may serve as the basis for writing an international standard.

2. Defining orthogonal and complementary measurements

2.1. Orthogonal measurements

Orthogonal measurements: *Measurements that use different physical principles to measure the same property of the same sample with the goal of minimizing method-specific biases and interferences.*

Note 1: Orthogonal measurements are used to minimize bias and interferences when considering a single type of sample analysed in the same conditions. The goal of orthogonal measurements is to determine the true value of a measurand.

Note 2: Two measurements cannot be orthogonal if they measure different properties. If trying to determine particle size, then measurements of particle size and particle composition cannot be orthogonal, since they are measuring different properties. They do not both provide information about the true value for the measurand of particle size.

Note 3: Orthogonal does not apply to measurements of different samples, such as aged vs. unaged samples. The value of a measurand for an aged and unaged samples may not be the same, which makes the concept of a true value nonsensical. Instead, orthogonal measurements should be used to determine the true value of the same sample (either the aged or unaged samples).

Note 4: The definition of orthogonal is not applicable to measurements that measure different dynamic ranges. For example, if two sizing measurements measure different size ranges, then they are not measuring the same particle populations since one of them is measuring bigger particles and the other is measuring smaller particles (e.g., dynamic light scattering is better suited for larger particles while DOSY NMR is better for smaller particles) [10].

Note 5: Sample preparation steps associated with measurements (such as the cryo-vitrification step required for cryo-TEM, the high dilution for NTA, the particle fractionation via AF4 prior to MALS measurements during AF4-MALS, etc.) can modify the properties of a sample. This must be taken into account during analysis.

Note 6: A measurement can be based on multiple physical principles. There may be two or more physical principles to be considered when assessing orthogonality of two measurements. An example is AF4-MALS which is based on the physical principles of diffusion and Rayleigh ratio. Two measurements may be orthogonal if they differ for at least one physical principle.

Note 7: The concept of orthogonality can only be applied to measurands for which there are two or more measurements that are based on different physical principles. If orthogonal methods to measure a property do not exist, then other strategies can be used to assess bias, such as use of better equipment to make the measurements, improving measurement reproducibility, an interlaboratory study or developing a reference material for calibration [11,12].

2.1.1. Discussion of orthogonal measurements

The flow chart in Fig. 1 can help to determine the orthogonality and complementarity of two measurements. The goal of orthogonal measurements is to determine the true value of a measurand. Two measurements can be orthogonal if they are used to measure the same attribute of the same sample, but the measurements are based on different physical principles. If two measurements are based on different physical principles, then their biases and interferences are likely to be different. In this way, orthogonal measurements provide different

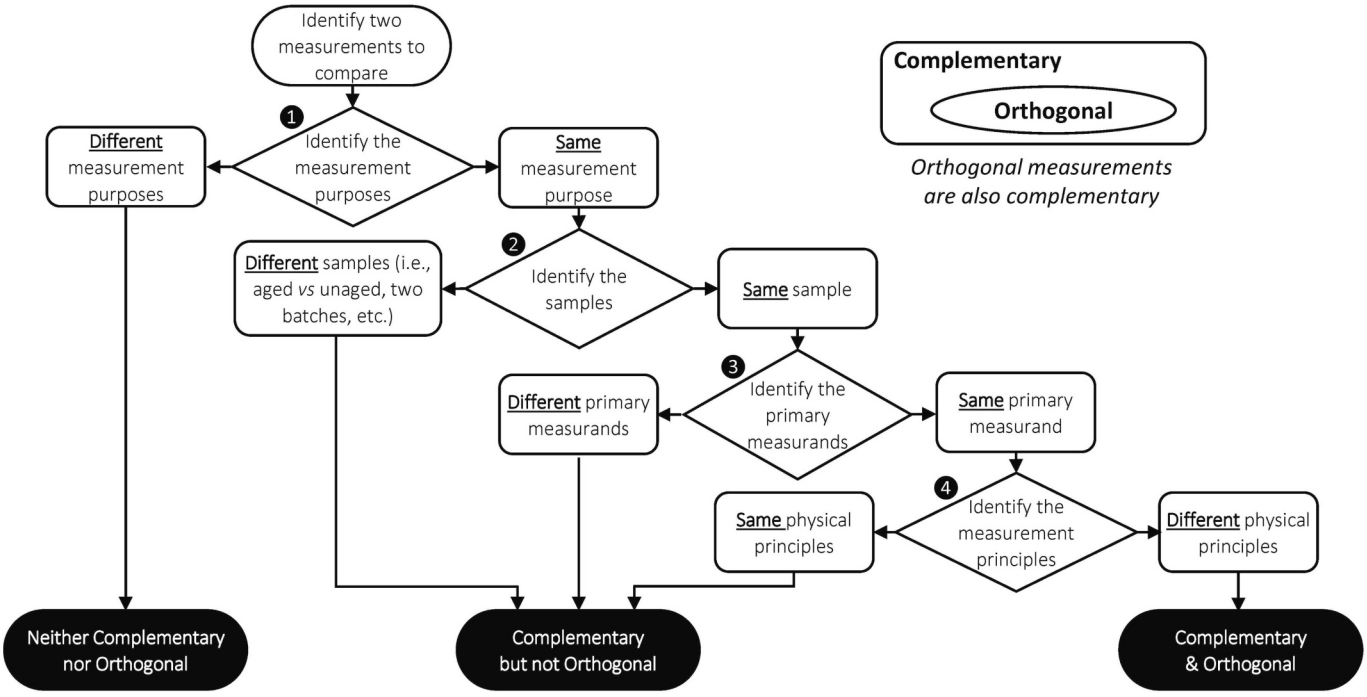

Fig. 1. Flow chart for the determination of complementary and/or orthogonal measurements based on the determination of (1) the measurement purposes; (2) the samples to be measured; (3) the primary measurands; and (4) the main physical principles behind the measurement. The flow chart is a guide to help users determine whether two measurements are (i) neither complementary nor orthogonal, (ii) complementary but not orthogonal, or iii) both complementary and orthogonal. Note that one condition must be met for two measurements to be complementary (same measurement purpose), while three additional conditions must be met for two measurements to be orthogonal (same samples, same primary measurands, and different measurement principles).

information about the same measurand to provide greater understanding of the measurand's true value.

The Guide to the Expression of Uncertainty in Measurement (GUM) explores orthogonality using the concepts of random and systematic effects on a measured value [13]. A random effect is an effect on a measurand where one cannot predict the next value. Random effects can be described by a probability density function. The uncertainty from random effects can be reduced by increasing the number of replicates measured. For systematic effects, such as interferences, one can predict the next value with some level of certainty. Systematic effects may be based on the physical principle behind the measurement. If the systematic effects are known, then it is possible to accommodate them in the measurement model. According to the GUM [13], an approach to evaluate a method for unknown biases is to test the product in the same experimental conditions *with orthogonal methods*. In fact, measurement bias can be defined as "an estimate of a systematic measurement error" [14].

Determining orthogonality is dependent on how users define four items: the measurement purpose, the samples, the measurands and the physical principles of the measurements. Given the same set of measurements, different scientists may arrive at different definitions for these four items. The concepts are not absolute and are open to interpretation and debate. Fig. S3 highlights challenges in defining the sample. For example, if measuring a sample's particle size by AUC and TEM, and the samples must be cryo-vitrified before TEM, then the samples may be considered the same and the measurements are orthogonal (Fig. S3a). However, if the effect of cryo-vitrification on particle size is being assessed by AUC, then measurements of cryo-vitrified and non-cryo-vitrified samples by AUC would not be orthogonal (Fig. S3b) since the samples are different. Similar examples for sample dilutions are addressed in Fig. S3c-e. For a product development team, deep discussion is required to align on definitions so that a consistent determination regarding orthogonality and complementarity can be made.

2.2. Complementary measurements

Complementary measurements: measurements that corroborate each other to support the same decision.

Note 1: All measurements that are orthogonal are also complementary. Not all complementary measurements are orthogonal (Fig. 1).

Note 2: Complementary measurements may measure the same property of the same sample, but within different dynamic ranges.

Note 3: Complementary measurements may measure different samples. Complementary measurements may assess the particle size of samples that are dispersed in different types of media or of aged versus unaged samples.

Note 4: The properties measured by complementary measurements do not need to be the same (such as measurements of drug release and aggregation propensity).

2.2.1. Discussion of complementary measurements

The definition of complementary measurements includes orthogonal measurements, but is broader in scope (Fig. 1). Orthogonal measurements determine the true value of a measurand through the use of measurements that are based on different physical principles. Complementary measurements are any measurements that are being conducted for the same measurement purpose. When the goal is characterization of a drug product for human use, measurements to assess quality, safety and efficacy may be considered complementary, since they are serving the same overall purpose. For example, a particle size measurement and a particle composition measurement, which are measurements of different drug product attributes, may be considered complementary if the purpose of both measurements is to assess safety and efficacy. Likewise, if the same measurement is performed on different samples, such as the stability assessment of aged versus unaged drug samples, then these measurements could also be considered complementary, if their purpose is to assess the stability of the drug product over time.

An example of measurements with different purposes that are not

complementary could be a measurement of atmospheric ozone to assess climate change versus a measurement of the particle size of a drug to assess safety and efficacy. However, if the measurement purpose for these measurements is broadened to "assessing and improving global health and safety", then these same two measurements could be considered complementary. Thus, it is important to carefully define the measurement purpose. Without carefully defined purposes, samples, measurands and measurement principles, it is difficult to achieve a consistent assessment of orthogonality and complementarity.

3. Examples

Examples of using the definitions to assess the orthogonality and complementarity of measurements of drug products containing nanomaterials are discussed below. Four measurands are discussed. Many of the examples focus on (i) particle size, but (ii) particle concentration, (iii) stability and (iv) shape are also addressed. These examples span a broad selection of nanoparticle classes, including virus-like particles (VLPs), liposomes, lipid-based nanoparticles (LNPs), and polymeric nanoparticles. To help with understanding the determination of orthogonality and complementarity, a flow chart is presented in Fig. 1. For each example, the measurements are graphically depicted in a measurand chart where the sample, the measurement process, the different measurands involved, the physical principles behind the measurement and the measurement purposes are identified (Fig. 2, Figs. S3-S6) [15]. The measurement principles of the particle sizing techniques are also explained in the Supplementary Material (Section 2).

3.1. Particle size distribution: Virus-like particles

An example for particle size measurements is shown in Fig. 2 (measurand chart) and Fig. 3 (measurement results). The product is a polydisperse VLP vaccine for the influenza virus. Measurements were performed according to the EUNCL in native buffer following synthesis (t0), as described in Supplementary Materials. Measurement techniques were selected according to the approach of incremental complexity [9]. DLS showed a PSD composed of one moderately polydisperse population (polydispersity index (PDI) of 0.2) with a peak size of ~150 nm hydrodynamic diameter. To understand if the moderate polydispersity was caused by (i) vesicles of different sizes, or (ii) the formation of multimers, two additional measurements (NTA and cryo-TEM) were performed after dilution in the same buffer (VLP native media). NTA is complementary but not orthogonal to DLS because they are based on the same physical principle, diffusion measured by the Brownian motion via the Stokes-Einstein equation (Fig. 2). However, cryo-TEM is orthogonal to DLS, because they are based on different physical principles: cryo-TEM detects electron contrast (nuclei density). cryo-TEM detected a smaller population of vesicles, of 25 nm to 50 nm, that was not evident in NTA or DLS. Moreover, cryo-TEM showed the presence of dimers and multimers formed by the larger population of vesicles, a consideration made possible only by direct observation of the particles after cryo-vitrification in their native buffer. NTA did not provide additional information compared to DLS, due to the bias of both light scattering-methods towards larger particles. Instead, a combination of orthogonal methods, such as DLS and cryo-TEM, was more informative [2]. Table 1 summarizes the application of orthogonal and complementary measurements to this example and the others in this section of the manuscript.

3.2. Particle size distribution: Nanoemulsion

In another example, several fundamentally different sizing methods (laser diffraction-LD, cryoTEM, DLS, NTA, DOSY NMR) were used to assess PSD of a nanoemulsion (Fig. S4) [16,17]. DLS and NTA are not orthogonal since they rely on the same physical principle (Brownian motion). However, DLS, LD, cryoTEM and 2D DOSY NMR are orthogonal to each other, since they use different physical principles to measure the same property (PSD) (Fig. 4, Table 2). DLS, LD, and DOSY NMR were ensemble measurements, which analyze the average size of the particles and all three are limited by low resolution. When used alone,

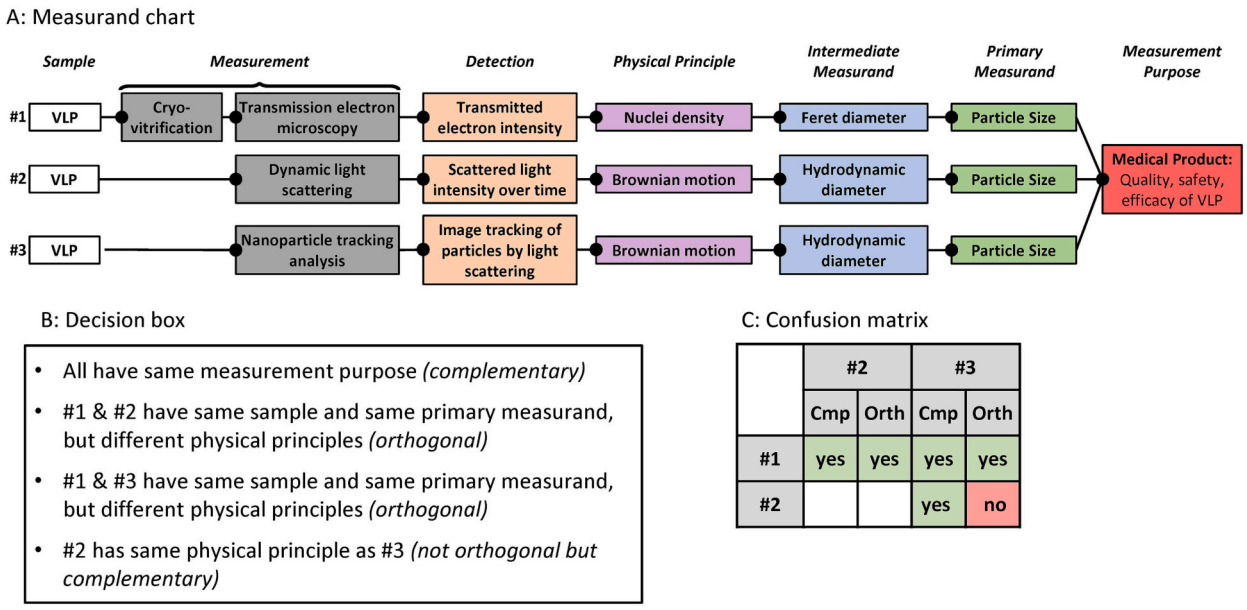

Fig. 2. A) Measurand chart**:** An example of a measurand chart for the measurement of the particle size distribution of a virus like particle sample (VLP) comparing TEM, DLS and NTA measurements made on the same sample in the same conditions for the same measurement purpose. From left to right. The white boxes are the sample, the grey boxes are the measurements, the orange boxes describe the detection principle used to transform the physical phenomenon into a measurable signal, the violet boxes indicate the physical principle of the measurement, the blue boxes are the "intermediate" measurand, the green boxes are the primary measurand (PSD in this example) and the red boxes are the measurement purpose (decision to be made using the data collected). B) Notes are provided in the decision box to explain the logic on how the decisions regarding complementarity and orthogonality were determined. C) Confusion matrix for the determination of orthogonality and complementarity for all pairwise comparisons of the measurements. Note: The relationships in the chart are not absolute and are open to discussion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

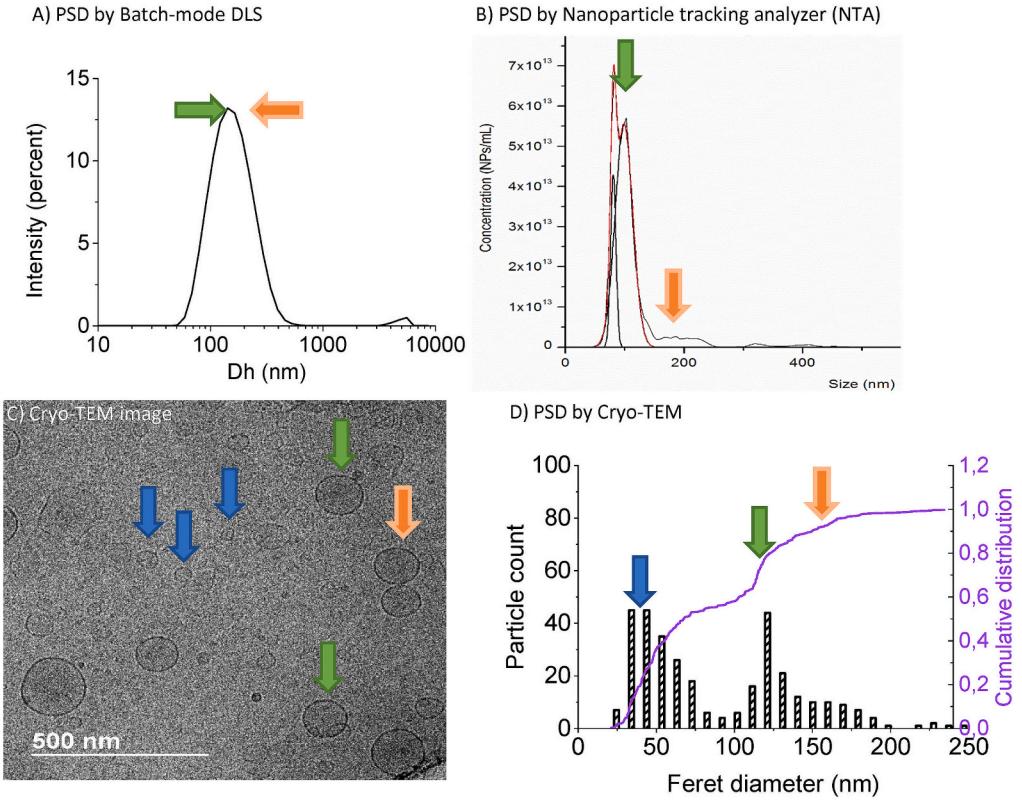

Fig. 3. Example of orthogonal techniques enabling a reduction in the uncertainty associated with the measurement of the PSD of a virus-like particle product (VLP 1). A) Representative DLS and B) NTA measurements due to the light scattering detection principle (not orthogonal to each other), are both biased towards larger particles, and do not detect the smaller 25 nm to 50 nm particle population. C—D) Cryo-TEM unmasks the systematic bias towards larger particles, and shows the presence of three main populations: (1) smaller vesicles of 25 nm to 50 nm (blue arrows), (2) larger vesicles of 100 nm to 150 nm (green arrows), and (3) dimers and multimers of isolated vesicles (orange arrows). PSD = particle size distribution. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Summary of how orthogonal and complementary measurements may reduce bias and interferences for the selected experimental examples.

Sample	Attribute	Measurement techniques	Possible bias source	Unmask bias? Combined information?	Reference
Virus like particles (VLP)	PSD (diameter: 1 nm to 400 nm), polydispersity	DLS	Overestimation of larger particles	*Unmask bias:* TEM may identify a smaller population of vesicles not visible by DLS and NTA. Combining measurements allows entire population of vesicles to be identified	Unpublished data
		Cryo-TEM	Artefacts due to cryo-vitrification, particles <300 nm may not be detected		
		NTA	Sample dilution, overestimation of larger particles		
Lipid-based nanoparticles loaded with mRNA or siRNA	Particle concentration and PSD (diameter: 30 nm to 300 nm)	NTA	Sample dilution	*Unmask bias:* Agreement in measured values reduces technique-related bias on measured particle concentration	[19,20]
		MALS (flow mode)	RI and shape information needed, sample recovery from AF4		
Liposomes (irinotecan)	Particle size	DLS	Spherical shape assumed	*Combined information* about particle shape and particle size could influence the safety and efficacy profile of the liposomes	[23]
	Particle size and shape (diameter: 30 nm to 300 nm)	Cryo-TEM	Artefacts due to cryo-vitrification		
		DLS-MALS in flow mode	Measurements in flow, sample recovery from AF4		
Polymeric NPs loaded with doxorubicin (drug)	Drug loading, drug release, particle size, stability	AUC with to UV-VIS detection at 490 nm	Instability of the particles leading to drug release during the measurement window.	*Unmask bias:* Agreement in measured values reduces technique-related bias and concerns of doxorubicin loss during ultracentrifugation *Combined information* about particle stability in plasma associated with increase in efficacy of nanoformulation (vs. free drug)	[23]
	Drug loading, drug release	Ultracentrifug-ation plus detection by HPLC-MS/MS	Drug loss during ultracentrifugation		
Nanoemulsions	Particle size distribution	DLS	Sensitivity to viscosity input and interferences from other excipients	*Combined information* about impact of dilution and excipient interference on size measurement of nanoemulsions	[16,17]
		LD	Smaller particles scatter at larger detector angle which can be difficult to measure		
		DOSY NMR	Measures only average diffusion coefficient		
		NTA	Sample dilution		
		CryoTEM	Sample preparation, biased towards smaller size fraction		
		AF4-MALS-DLS	Measurement in flow, sample recovery from AF4		

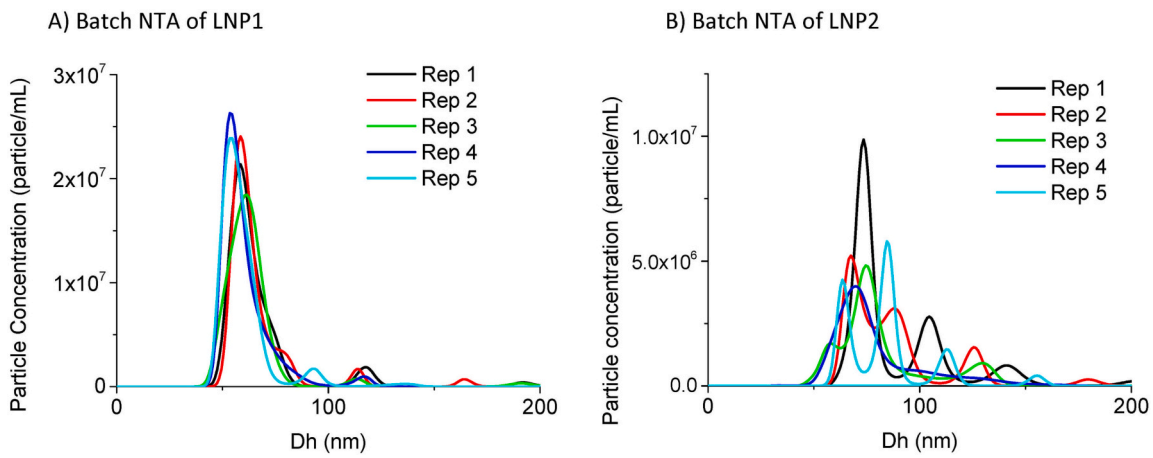

Fig. 4. Complementary techniques to measure particle concentration of two lipid nanoparticles (LNP) for mRNA delivery. (A) LNP1 and (B) LNP2 measured by batch NTA showing the variability obtained by measuring five replicates of the same sample in a time window of 10 min. Variability of the PSD in LNP2 indicated instability of the sample (changes in particle size and concentration) during the measurement.

Table 2
Example of orthogonal and complementary particle size measurements of a nanoemulsion (measured as described [16,17]).

Technique	Measurand	Measured value ± SD
DLS	Dh (Z-Average)	117.9 ± 2.0 nm
LD	Dv50	73.1 ± 1.5 nm
NTA	D50	107.7 ± 1.3 nm
Cryo-TEM	Number mean	26.6 ± 14.3 nm
2D DOSY NMR	^{1}H NMR intensity averaged	70.5 ± 3.5 nm

each method was not sufficient to address particle size uncertainty and bias. For example, DLS results were intensity-based and biased towards larger sizes. LD results trended smaller relative to DLS. Furthermore, DOSY NMR determined the average diffusion coefficient, so the results were only able to capture the average size instead of a distribution. Two other methods (NTA and cryoTEM) produced number-weighted size results. Although the methods addressed the uncertainty/bias associated with the distribution (due to better size resolution), both NTA and cryoTEM were limited by what can be 'seen' as well as the statistical power (number of particles analysed). Individually, neither NTA nor cryoTEM could address the uncertainty associated with accuracy. Yet, when all five methods were combined to analyze the PSD, especially under the influence of dilution, they all produced similar conclusions about the effect of polymer excipient interferences (carbomer), and all were useful for decision-making [16,17]. Further evaluation of the same sample using AF4-MALS-DLS revealed the presence of two populations of particles. The more abundant population had particles smaller than 100 nm and the smaller population had particles larger than 100 nm. AF4-MALS-DLS results provided reasonable agreement with observations from the previous five methods, further improving confidence in results. In this example, use of both complementary and orthogonal methods improved the understanding of the particle size that allowed for better decision-making regarding control of product quality.

3.3. Particle concentration and aggregation propensity: Lipid based nanoparticles (LNPs)

Measurement of particle concentration associated with the measurement of particle size may be a useful indicator of physical stability of drug products containing nanomaterials. If reliable methods are used, measurement of particle concentration combined with (i) the evaluation of the particle aggregation propensity and (ii) the chemical stability could be used to select appropriate storage conditions, define the reconstitution protocol, and/or define the product dilution prior to administration to patients. This is pertinent in the assessment of the stability of LNP formulations used for the delivery of mRNA as vaccines against COVID-19 [18]. An example of measuring storage stability of LNP loaded with siRNA by AF4-MALS by combining measurements of aggregation propensity and concentration for aged and unaged samples has been described (Fig. S5) [19,20].

Particle concentration measurements are often challenged by high variability among different techniques which may be associated with systematic biases [21]. For example, samples prepared for NTA may suffer from instability due to the high dilution of the stock (> 1000-fold). High dilution may cause particle destabilisation and misleading results. This is shown in Fig. 4A,B where the concentration of two LNP formulations loaded with the same mRNA but different lipid compositions (LNP1 and LNP2) have been analysed by NTA after a 10,000 x dilution. In the case of LNP2, the measured concentration dropped by half in <10 min (time necessary to perform 5 replicate measurements), while LNP1 was stable. This shows how NTA is not suitable to measure the particle concentration and PSD of LNP2.

It is often difficult to rule out a priori the impact of dilution on LNP stability, since it likely depends on complex variables such as the chemical composition of the LNP, physical arrangement of the LNP, surface charge, and dispersion media. Testing orthogonal or complementary light scattering approaches that require lower dilution of the samples prior to measurements, such as AF4-MALS or multi-angle DLS [19,20], can be used to validate the results obtained by NTA. At the same time, both approaches are based on measurement of particle concentration derived by light scattering and require prior knowledge of particle shape and composition. The results may be biased by the choice of the light scattering model. To get accurate concentrations, the chemical composition (mRNA vs lipid ratio) must be known, so that an accurate refractive index can be determined [19].

An example comparing results obtained by batch NTA and AF4-MALS for the measurement of the PSD and particle concentration for another batch of LNP with the same lipid composition of LNP1 (named LNP3) is reported [19]. The measurand charts and the confusion matrix applying to these examples are in Fig. S5. This combination of methods highlights concerns that the results are affected by intrinsic measurement bias due to the sample preparation, e.g., too high dilution in the case of NTA, or data treatment when concentration is measured indirectly by AF4-MALS (e.g., an incorrect refractive index used to derive the particle concentration values from light scattering data or variability induced by the model used to treat light scattering data).

3.4. Chemical and physical stability in physiological media: Polymeric nanoparticles

After entering circulation, nanomaterials interact with plasma proteins, gaining a protein corona which influences their biodistribution [22]. The protein-particle interaction can destabilize structure, induce size changes, and influence the API release [22]. Stability measurements have two levels of complexity. First, the free protein fraction must be separated from the protein-particle complexes, which increases risk of bias. Therefore, confirmation of results with an orthogonal measurement is desired. Second, protein-particle interactions affect both the chemical and physical properties of the particles acting on different CQAs at the same time in a complex and interconnected way. Therefore, to get a full understanding of the particle stability, complementary approaches are needed to measure different attributes under the same conditions, including, for example, changes in particle size and drug release.

An example of how multiple techniques can be used to understand stability in plasma has been described [23]. AUC and high performance liquid chromatography-mass spectrometry/mass spectrometry (HPLC-MS/MS) were used to assess free API (vs the encapsulated API) and API release. In this case, the focus is on the measurement *of the same primary measurand*: the concentration of free doxorubicin. The results obtained from the drug release measurements (consisting of an ultrafiltration step to separate the free drug and the protein-bound free drug from liposome-bound fractions followed by LC-MS/MS detection of the drug in the different fractions [24–26]) were corroborated by AUC measurements. The former approach suffers from a possible bias caused by loss of the API on the filtering devices used in ultrafiltration [2,27,28]. AUC performs the separation and drug quantification in a single step which reduces the risk of bias in drug quantification and helps to support the obtained drug release profile. In this case, both techniques confirmed an unexpected burst (very fast) of released API.

AUC was also applied as a complementary technique to LC-MS/MS to measure the size changes of the particles in plasma in addition to the drug release [27,29]. After using an additional separation step between particles and proteins prior to sizing detection, AUC and AF4 can measure particle size at high resolution by eliminating interferences generated by the free protein fraction. Both measurements detected an increase in particle size, demonstrating the presence of NP-protein interactions that triggered the API release mechanism.

3.5. Particle shape of liposomal formulations

An example of the application of orthogonal and complementary measurements to particle shape of liposomal formulations is presented in Supplementary (Section 4, Fig. S6).

4. Discussion

The goals of this paper are (1) to propose definitions for “orthogonal” and “complementary” measurements, (2) to provide a practical framework for assessing the orthogonality of two measurements that is in line with existing guidances and (3) to apply the definitions and framework to examples of measurements of drug products containing nanomaterials. The definitions and the framework for assessing orthogonality may be useful in fields beyond drug products containing nanomaterials, such as toxicology, material science, nanomaterials or biology, where complex measurands are measured and integrated for the understanding of a product or a biological process. Although this paper does not carry the same weight as a consensus standard, it is an important first step to introduce new concepts that were developed by an international team of pharmaceutical product development stakeholders representing a wide range of organizations including metrology institutes, characterization laboratories, policy makers and regulatory agencies. This paper can help users assess the orthogonality and complementarity of measurements in the transition period where no consensus standards are available, stimulate further discussion within the community and serve as the basis for writing an international consensus standard.

5. Conclusions

In this work, definitions are proposed for orthogonal and complementary measurements to clarify their use in characterizing nano-enabled drug products. Orthogonal measurements are based on different physical principles and can be used to address unknown systematic measurement bias to gain a better understanding of the true value of a product attribute. Complementary measurements include a broader scope of methods that reinforce each other to support a common decision. Integration of information garnered from complementary methods is a critical step towards evaluation of complex measurands, such as the evaluation of product quality. Examples of measurements of physical properties of drug products containing nanomaterials were used to illustrate how orthogonality and complementarity can be assessed with the proposed definitions. The application of the orthogonality/complementarity framework to the investigation of nanomaterial drugs has the potential to significantly advance the testing of pharmaceutical products, helping to bring novel medicines to clinic more rapidly.

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Author contribution

All the authors contributed to the definitions and the writing of the article. FC and CGS coordinated the writing of the manuscript. FC and JP performed the unpublished experimental measurement reported in this article. All authors have approved the manuscript.

Declaration of Competing Interest

The authors declare no competing interests.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

The Supplementary Material contains a glossary, descriptions of the methods used to collect the data provided in the examples, an additional example addressing particle shape of liposomes, additional figures and additional references. Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2022.12.049.

References

[1] K.M. Tyner, N. Zheng, S. Choi, X. Xu, P. Zou, W. Jiang, C. Guo, C.N. Cruz, How has CDER prepared for the nano revolution? A review of risk assessment, regulatory research, and guidance activities, AAPS J. 19 (2017) 1071–1083, https://doi.org/10.1208/s12248-017-0084-6.

[2] FDA Guidance for Drug Products, Including Biological Products, that Contain Nanomaterials - Guidance for Industry, U.S. Food and Drug Administration, 2017. Accessed July 31, 2022: https://www.fda.gov/media/157812/download.

[3] FDA Draft Guidance - Development of Therapeutic Protein Biosimilars: Comparative Analytical Assessment and Other Quality-Related Considerations - Guidance for Industry, U.S. Food and Drug Administration, 2019. Accessed July 31, 2022: https://www.fda.gov/media/159261/download.

[4] FDA Guidance - Bioanalytical Method Validation - Guidance for Industry, U.S. Food and Drug Administration, 2018. Accessed July 31, 2022: https://www.fda.gov/media/70858/download.

[5] International Conference on Harmonisation, Validation of Analytical Procedures: Q2(R2) Draft Version, Accessed December 9, 2022: https://database.ich.org/sites/default/files/ICH_Q2-R2_Document_Step2_Guideline_2022_0324.pdf, 2022.

[6] W. Li, J.L. Kerwin, J. Schiel, T. Formolo, D. Davis, A. Mahan, S.A. Benchaar, Structural elucidation of post-translational modifications in monoclonal antibodies, in: State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization Volume 2. Biopharmaceutical Characterization: The NISTmAb Case Study, American Chemical Society, 2015, pp. 119–183, https://doi.org/10.1021/bk-2015-1201.ch003.

[7] NIST Reference Material 8671 - NISTmAb, Humanized IgG1κ Monoclonal Antibody. Reference Material Information Sheet, National Institute of Standards & Technology, Gaithersburg, MD, 2022. Accessed July 31, 2022.

[8] Pubmed search for "Title" containing (characterisation OR characterization) AND (complementary OR orthogonal), Accessed September 2021: https://pubmed.ncbi.nlm.nih.gov/.

[9] F. Caputo, J. Clogston, L. Calzolai, M. Rösslein, A. Prina-Mello, Measuring particle size distribution of nanoparticle enabled medicinal products, the joint view of EUNCL and NCI-NCL. A step by step approach combining orthogonal measurements with increasing complexity, J. Control. Release 299 (2019) 31–43, https://doi.org/10.1016/j.jconrel.2019.02.030.

[10] S.M. Patil, D.A. Keire, K. Chen, Comparison of NMR and dynamic light scattering for measuring diffusion coefficients of formulated insulin: implications for particle size distribution measurements in drug products, AAPS J. 19 (2017) 1760–1766, https://doi.org/10.1208/s12248-017-0127-z.

[11] G.H. White, Basics of estimating measurement uncertainty, Clin. Biochem. Rev. 29 (2008) S53–S60. Accessed October 14, 2022: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2556585/.

[12] NIST/SEMATECH e-Handbook of Statistical Methods, Section 2.1.1.3. Bias and Accuracy, 2022, https://doi.org/10.18434/M32189. Accessed October 14, 2022:.

[13] Evaluation of Measurement Data - Guide to the Expression of Uncertainty in Measurement (GUM); Joint Committee for Guides in Metrology (JCGM) 100:2008 (E); JCGM, Accessed July 31, 2022, https://www.bipm.org/utils/common/documents/jcgm/JCGM_100_2008_E.pdf, 2008.

[14] International Vocabulary of Metrology - Basic and General Concepts and Associated Terms (VIM), 3rd ed.; Joint Committee for Guides in Metrology (JCGM), Accessed July 31, 2022: https://www.bipm.org/utils/common/documents/jcgm/JCGM_200_2012.pdf, 2012.

[15] D. Arora, G. Babakhanova, C.G. Simon, Tissue engineering measurands, ACS Biomater. Sci. Eng. 6 (2020) 5368–5376, https://doi.org/10.1021/acsbiomaterials.0c00475.

[16] P.E. Petrochenko, N. Pavurala, Y. Wu, S. Yee Wong, H. Parhiz, K. Chen, S.M. Patil, H. Qu, P. Buoniconti, A. Muhammad, S. Choi, D. Kozak, M. Ashraf, C.N. Cruz, J. Zheng, X. Xu, Analytical considerations for measuring the globule size distribution of cyclosporine ophthalmic emulsions, Int. J. Pharm. 550 (2018) 229–239, https://doi.org/10.1016/j.ijpharm.2018.08.030.

[17] H. Qu, J. Wang, Y. Wu, J. Zheng, Y.S.R. Krishnaiah, M. Absar, S. Choi, M. Ashraf, C. N. Cruz, X. Xu, Asymmetric flow field flow fractionation for the characterization of globule size distribution in complex formulations: a cyclosporine ophthalmic emulsion case, Int. J. Pharm. 538 (2018) 215–222, https://doi.org/10.1016/j.ijpharm.2018.01.012.

[18] L. Schoenmaker, D. Witzigmann, J.A. Kulkarni, R. Verbeke, G. Kersten, W. Jiskoot, D.J.A. Crommelin, mRNA-lipid nanoparticle COVID-19 vaccines: structure and stability, Int. J. Pharm. 601 (2021), 120586, https://doi.org/10.1016/j.ijpharm.2021.120586.

[19] R. Mildner, S. Hak, J. Parot, A. Hyldbakk, S.E. Borgos, D. Some, C. Johann, F. Caputo, Improved multidetector asymmetrical-flow field-flow fractionation method for particle sizing and concentration measurements of lipid-based nanocarriers for RNA delivery, Eur. J. Pharm. Biopharm. 163 (2021) 252–265, https://doi.org/10.1016/j.ejpb.2021.03.004.

[20] F. Caputo, C. Sieg, Meeting regulatory needs in the characterization of lipid nanoparticles for RNA delivery via FFF-MALS, Column 17 (7) (2021) 9–15. Accessed July 31, 2022: https://cdn.sanity.io/files/0vv8moc6/chroma/c4e759b30ae49ad74c6a8c1e0d13201f2f1c1f98.pdf/Vol17_Issue7_TheColumn_July2021_Europe%26Asia.pdf.

[21] E.J. Petersen, A.R.M. Bustos, B. Toman, M.E. Johnson, M. Ellefson, G.C. Caceres, A. L. Neuer, Q. Chan, J.W. Kemling, B. Mader, K. Murphy, M. Roesslein, Determining what really counts: modeling and measuring nanoparticle number concentrations, Environ. Sci.: Nano. 6 (2019) 2876–2896, https://doi.org/10.1039/C9EN00462A.

[22] V. Francia, R.M. Schiffelers, P.R. Cullis, D. Witzigmann, The biomolecular Corona of lipid nanoparticles for gene therapy, Bioconjug. Chem. 31 (2020) 2046–2059, https://doi.org/10.1021/acs.bioconjchem.0c00366.

[23] F. Caputo, D. Mehn, J.D. Clogston, M. Rösslein, A. Prina-Mello, S.E. Borgos, S. Gioria, L. Calzolai, Asymmetric-flow field-flow fractionation for measuring particle size, drug loading and (in)stability of nanopharmaceuticals. The joint view of European Union Nanomedicine Characterization Laboratory and National Cancer Institute - Nanotechnology Characterization Laboratory, J. Chromatogr. A 1635 (2021), 461767, https://doi.org/10.1016/j.chroma.2020.461767.

[24] S. Skoczen, S.E. McNeil, S.T. Stern, Stable isotope method to measure drug release from nanomedicines, J. Control. Release 220 (2015) 169–174, https://doi.org/10.1016/j.jconrel.2015.10.042.

[25] S. Stern, S. Skoczen, NCL Method PHA-2 (Version 2.0)., NCI Hub, 2020, https://doi.org/10.17917/8EJ1-9P65.

[26] S.L. Skoczen, S.T. Stern, Improved ultrafiltration method to measure drug release from nanomedicines utilizing a stable isotope tracer, Methods Mol. Biol. 1682 (2018) 223–239, https://doi.org/10.1007/978-1-4939-7352-1_19.

[27] D. Mehn, P. Lavicoli, N. Cabaleiro, S.E. Borgos, F. Caputo, O. Geiss, L. Calzolai, F. Rossi, D. Gilliland, Analytical ultracentrifugation for analysis of doxorubicin loaded liposomes, Int. J. Pharm. 523 (2017) 320–326, https://doi.org/10.1016/j.ijpharm.2017.03.046.

[28] S. Gioria, F. Caputo, P. Urbán, C.M. Maguire, S. Bremer-Hoffmann, A. Prina-Mello, L. Calzolai, D. Mehn, Are existing standard methods suitable for the evaluation of nanomedicines: some case studies, Nanomedicine (London) 13 (2018) 539–554, https://doi.org/10.2217/nnm-2017-0338.

[29] D. Mehn, R. Capomaccio, S. Gioria, D. Gilliland, L. Calzolai, Analytical ultracentrifugation for measuring drug distribution of doxorubicin loaded liposomes in human serum, J. Nanopart. Res. 22 (2020) 158, https://doi.org/10.1007/s11051-020-04843-5.