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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

F. Caputo[a,b], D. Mehn[c], J.D. Clogston[d], M. Rösslein[e], A. Prina-Mello[f], S.E. Borgos[a], S. Gioria[c], L. Calzolai[c,*]

[a] Department of Biotechnology and Nanomedicine, SINTEF Industry, Trondheim, Norway
[b] Univ. Grenoble Alpes, CEA, LETI, F-38000 Grenoble, France
[c] European Commission, Joint Research Centre (JRC), Ispra, Italy
[d] Nanotechnology Characterization Laboratory (NCL), Cancer Research, Technology Program, Leidos Biomedical Research, Inc., Frederick, National Laboratory for Cancer Research, Frederick, MD 21702, USA
[e] Swiss Federal Laboratories for Materials Research and Testing, Laboratory for Particles—Biology Interactions, EMPA, Lerchenfeldstrasse 5, St. Gallen CH-9014, Switzerland
[f] LBCAM, Department of Clinical Medicine, Trinity Translational Medicine Institute, Trinity College Dublin, Dublin, Ireland

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A B S T R A C T

Asymmetric-flow field-flow fractionation (AF4) has been recognized as an invaluable tool for the characterisation of particle size, polydispersity, drug loading and stability of nanopharmaceuticals. However, the application of robust and high quality standard operating procedures (SOPs) is critical for accurate measurements, especially as these complex drug nanoformulations are most often inherently polydisperse. In this review we describe a unique international collaboration that lead to the development of a robust SOP for the measurement of physical-chemical properties of nanopharmaceuticals by multi-detector AF4 (MD-AF4) involving two state of the art infrastructures in the field of nanomedicine, the European Union Nanomedicine Characterization Laboratory (EUNCL) and the National Cancer Institute-Nanotechnology Characterisation Laboratory (NCI-NCL). We present examples of how MD-AF4 has been used for the analysis of key quality attributes, such as particle size, shape, drug loading and stability of complex nanomedicine formulations. The results highlight that MD-AF4 is a very versatile analytical technique to obtain critical information on a material particle size distribution, polydispersity and qualitative information on drug loading. The ability to conduct analysis in complex physiological matrices is an additional very important advantage of MD-AF4 over many other analytical techniques used in the field for stability studies. Overall, the joint NCI-NCL/EUNCL experience demonstrates the ability to implement a powerful and highly complex analytical technique such as MD-AF4 to the demanding quality standards set by the regulatory authorities for the pre-clinical safety characterization of nanomedicines.

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1. Introduction

Nanopharmaceuticals are medicinal products where the active pharmaceutical ingredient (API) is encapsulated in – or associated to – biocompatible nanoparticles (NPs) in order to improve the API safety and/or efficacy profiles compared to the free drug. The first nanomedicine introduced to the clinic was liposomal doxorubicin (Doxil®) in 1995, with notably reduced cardiotoxicity compared to free doxorubicin. Very recently, the nanomedicine field has received significant attention in relation to the ongoing Covid-19 pandemic, where several of the frontrunner vaccine candidates use nanoparticles to carry either the antigen or the messenger RNA.

Physical-chemical properties specific to nanopharmaceuticals, such as particle size and size distribution, morphology, drug load-

* Corresponding author.
E-mail address: Luigi.CALZOLAI@ec.europa.eu (L. Calzolai).

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ing and stability in biological media are key attributes that influence safety and efficacy profile of the formulation. For this reason, regulatory agencies, including the US Food and Drug Administration (FDA) and the European Medical Agency (EMA), have published multiple regulatory guidance documents requiring the accurate assessment of these properties as part of the pre-clinical characterisation of any nano-formulation and also for the evaluation of their bioequivalence when in generic formulations [1–8]. In this context, the development of reliable pre-clinical characterisation strategies and suitable standard protocols are critical steps to sustain the successful industrial development and clinical translation of nanopharmaceuticals. The US National Cancer Institute Nanotechnology Characterization Laboratory (NCI-NCL) and, since 2015, the European Union Nanomedicine Characterisation Laboratory (EUNCL) have supported the pharmaceutical community by providing an integrated, holistic and comprehensive pre-clinical testing approach, including the measurement of physical-chemical properties and formulation stability in biological settings.

In the context of physical-chemical characterisation, the EUNCL and the NCI-NCL have proposed and published a three steps approach of incremental complexity [9,10]. This summarise in: step 1 is a quick preliminary check for sample integrity and stability by low resolution techniques (pre-screening) aimed at detecting early failures without engaging in more complex, time-consuming, and expensive techniques. In this preliminary "pre-screening" step, size measurements are performed with batch dynamic light scattering (DLS) whereas total drug loading can be evaluated by HPLC-UV-VIS or LC-MS/MS. Once step 1 is satisfactorily accomplished, the combination of complementary high resolution techniques is suggested for a robust assessment of sample behaviour in simple buffers, e.g., for understanding batch-to-batch variability, or to assess storage stability (step 2), and then subsequently in complex biological media (step 3).

Among the high resolution analytical techniques considered for steps 2 and 3, asymmetric-flow field-flow fractionation coupled with multiple detectors (MD-AF4) is one of the most versatile for the characterisation of particle size, polydispersity, and stability of complex nanopharmaceuticals [9,11–13]. The coupling of a gentle separation of particles according to their size prior to detection by using multiple, orthogonal detection principles, improves the resolution and accuracy of the analysis, thus allowing the measurement of very complex polydisperse samples. Furthermore, the technique allows post-detector collection of the eluting fractions and subsequent analysis by further offline techniques, e.g. for chemical composition. MD-AF4 has been extensively used by NCI-NCL and EUNCL for different purposes, including the measurement of particle size distribution and nanocarrier physical stability in biological media, for the evaluation of drug loading, and to check stability and kinetics (e.g. burst release of the active pharmaceutical principle) in blood plasma [11].

In this review we describe the process behind the development of a standard operating procedure for MD-AF4 measurements. This representing the successful example on the know-how and tech transfer of protocols between NCI-NCL and EUNCL laboratories. Moreover, we will discuss how the acquired knowledge by the two infrastructures, recently allowed initiating the process for a new standard test method in collaboration with ASTM International focalized on the "Physical characterization of liposomal drug formulations using multi-detector asymmetrical-flow field flow fractionation"; currently under finalization within the ASTM E56.02 committee. In the second part of the review, we will present three case studies with the aim to demonstrate the versatility of MD-AF4 in the analysis of nanopharmaceuticals. Overall, it is our intention to highlight: (i) the potential of AF4 coupled with on-line sizing detectors such as DLS and MALS to measure size distribution, and obtain morphological information (e.g. shape distribution) of polydisperse samples; and (ii) the use of MD-AF4 to study the drug exchange between liposomal vesicles and multilamellar vesicles. The MD-AF4 results will be compared to the current and most commonly used characterization methods; DLS and TEM to measure size and size distribution, and ultrafiltration with post HPLC or LC-MS analysis and analytical ultracentrifugation to measure drug stability and release (free and encapsulated drug ratios) in complex media. In addition, we will illustrate how these measurements (e.g. transmission electron microscopy, dynamic light scattering, analytical ultracentrifugation) can support, complement and validate the MD-AF4 results.

1.1. The principle of MD-AF4 method for nanopharmaceutical fractionation and analysis

The theory, sample applicability, and profile optimization for AF4, as well as a comparison to other chromatographic techniques, have been reviewed by many authors [11,12,14–16]. MD-AF4 has been successfully applied for the analysis of numerous nanopharmaceuticals, including liposomes [17–20], lipid-based nanoparticles [9], virus-like particles [21], polymeric NPs, but also extracellular vesicles [22], nano-crystals, inorganic oxide and gold nanoparticles [12,15,16].

AF4 separation is achieved by establishing a parabolic laminar flow profile of the liquid mobile phase in a thin channel, without the need for a stationary phase, thus being a very powerful tool for organic nanoparticles such as liposomes, lipid-based nanoparticles, extracellular vesicles and polymeric particles which are commonly used in nanomedicine. Particles of different sizes exit the separation channel at different times, starting from the smallest size to the largest.

Multiple detectors can be added downstream of the AF4 separation step, including sizing detectors such as dynamic light scattering (DLS) or multi-angle static light scattering (MALS), which allows the measurement of particle size with great accuracy. By combining results obtained by both in-line MALS and DLS detection, the radius of gyration (Rg) and hydrodynamic radius (Rh), respectively, can be measured simultaneously and used to calculate the shape factor parameter (ρ = Rg/Rh) [11,16,23,24], which is indicative of particle shape and morphology. Upon protein binding, the shape factor can be a useful tool in anticipating the *in vivo* behaviour of the formulation [11]. Online measurements performed with concentration detectors (such as UV-VIS, ICP-MS and/or refractive index) can allow the evaluation of chemical composition and drug loading [11,19]. Interestingly, in cases where the loaded active pharmaceutical ingredient (API) has a specific absorption at a wavelength where the nanoparticle components do not absorb, a UV-VIS absorbance measurement could also be used to estimate drug release and/or drug exchange between particles of different sizes.

One of the main challenges in the physical-chemical characterisation of nanopharmaceuticals is the evaluation of their behaviours in biological media, for example the (in)stability induced by the interaction of nanoparticle drug deliver carriers with plasma proteins. The nanoparticle-protein mixture is a very challenging system to measure by batch techniques, such as the widely used batch mode DLS. The introduction of the AF4 separation step, allows separation of the free proteins from the nanoparticles prior to the analysis, significantly improving the measurement resolution [9,13,23]. For this reason, whenever possible MD-AF4 is the technique of choice for the analysis of physical stability in complex biological media.

Despite being extremely useful for many NP formulations, MD-AF4 has some disadvantages that have limited the exploration of its full potential in the pharmaceutical sector until now [10]. First of all, the ideal elution conditions are often nanoparticle specific

and method optimization can be laborious, similarly to conventional chromatography. There are certain important criteria to be considered for a method for the analysis of nano-objects using asymmetrical-flow and centrifugal field-flow fractionation to be acceptable, as reported by the ISO/TS 21362 [25]. In particular, the particles should not be destabilized during the measurement, which could happen due to dilution or if a too strong crossflow is applied. In addition, at least more than 70% of the injected particles should be eluted as size separated fraction from the channel.

Sample loss due to particle-membrane interaction is one of the main limitations for some types of nanoparticles, especially in the case of positively charged ones. Moreover, some authors reported less accurate size measurements by using online DLS detector for particles larger than 250 nm [17]. Therefore, when performing MD-AF4, it is strongly recommended to follow an SOP which includes some critical quality checks to be aware of the possible sample loss in the channel, and to ensure appropriate analysis of the data obtained by light scattering [26]. Moreover, when dealing with new type of samples, it is good practice to compare the results with orthogonal high-resolution techniques, such as the direct visualization of the particles by electron microscopy. However, when an elution method for specific NPs platforms is successfully developed and if a robust SOP is followed, MD-AF4 is a powerful tool for synthesis optimization, quality control and monitoring the stability of the multiple classes of nanopharmaceuticals in complex biological media [17].

2. The path from a standard operating procedure to an international standard

In 2015, the EUNCL project set the ground as unique first European Union Horizon2020 infrastructure project with fully integrated US partner (NCI-NCL), setting the stage for intercontinental collaboration. At that point, NCI-NCL had more than 10 years of experience in translational research of nanopharmaceuticals and had characterised more than 400 different potential NP based medical products using various physical and chemical techniques, *in vitro* assays, and *in vivo* studies. A collection of more than 30 standard operating procedures (SOPs) was available, which in the meantime has been expanded considerably (https://ncl.cancer.gov/resources/assay-cascade-protocols). MD-AF4 was already then proven to be a powerful method for analysis of size and size distribution in polydisperse populations of medical nanoparticles.

2.1. Introduction of method development SOP for reliable use of a highly complex analytical method

Historically, through their first decade of operation, NCI-NCL developed an integrated approach looking at a given nanopharmaceutical with complementary methods. This approach allowed the NCI-NCL team a holistic view at the different aspects of the potential medical product [27–29]. As an example, they would relate a rather complex size distribution of the NP determined by MD-AF4 with notable *in vitro* assay results and complementary physical-chemical analysis such as particle size measurement by cryo-TEM. Within the EUNCL collaborative framework, NCI-NCL shared SOPs and know-how with the EUNCL distributed infrastructure [23]; followed by hands-on and optimized training at the NCI-NCL premises in Frederick (MD, USA). Not only their huge experience with individual techniques for the characterisation of nanopharmaceuticals and their large collection of SOPs, but also their integrated approach in characterization were important aspects of this knowledge transfer [13]. During this process the specialist of EUNCL, in discussion with the NCI-NCL, decided to expand the complex characterization scope by drafting *advance method development* SOPs for all those analytical techniques, which require an adjustment and optimisation phase prior to the actual measurements on any given nanomedicine or nano-objects are conducted (http://www.euncl.eu/about-us/assay-cascade/). As a general principle of guidance, the *advanced method development* SOPs describe in detail the optimisation process, which is important to achieve for setting the best measurement conditions with specified number of minimum quality control and acceptance criteria to allow the actual measurement series to be conducted. An example is the development of the SOP focusing on MD-AF4 measurements [26]. The step-by-step method development and data analysis procedure for light scattering (LS) data are reported in Fig. 1. Critical steps during method development includes: (i) optimizing operating conditions to obtain an acceptable sample recovery (>70%), (ii) assessment of measurement repeatability, (iii) correct procedures for calibration and normalization of the light scattering detector and (iv) quality control checking of the whole instrumental setting with suitable standards for both size and molecular weight; which are in line with ISO/TS 21362. Provided that all those criteria are fulfilled, the standard operating procedure (SOPs) describes how to reliably study various critical attributes of the nanopharmaceutical formulations including: (i) the measurement of size distribution of nanoparticles in physiological buffers; (ii) change in nanoparticle size distribution after protein binding (NP-proteins interaction); (iii) molecular weight (MW) of NPs components (for polymeric carriers); (iv) release of free coatings/surfactants (e.g. PEG) from the surface of the particles. The advanced MD-AF4 SOP was tested by means of inter laboratory comparison (ILC) on representative nanopharmaceutical formulations (as described in the following paragraph) after which it was made available for the whole community.

2.2. Inter-laboratory testing as the first step to confirm applicability for regulatory acceptance

Liposomal drugs are still the most commonly nanomedicine formulations reaching clinical trials; these are composed of a lipid bilayer encapsulating an aqueous core through non-covalent lipid interactions. A publication by the European Nanomedicine Technology Platform (ETPN) recently reported that liposomal drugs constitute >50% of the currently ongoing clinical trials around the world [30]. Moreover, the criteria to consider for assessing biosimilarity of generic liposomal drug formulations is a topic of intense discussion (and concerns) for regulatory agencies and the pharmaceutical sector alike [31]. These factors both contribute to the immense interest and importance of liposomes in the field of nanomedicines. In this context, a liposomal doxorubicin research grade formulation, possessing the same physical-chemical properties of the first approved nanomedicine formulation (Doxil) was selected for a qualification campaign of the EUNCL infrastructure and of their SOPs. The different SOPs were tested by performing ILC studies (involving both EUNCL and NCI-NCL partners) and/or by comparing the obtained results with complementary techniques. For the qualification of the advanced MD-AF4 SOP, two different measurement platforms were used for MD-AF4 analytical technique comparison. The acquired results were then compared with orthogonal techniques, including batch mode DLS and cryo-TEM. The details of the AF4 setup are reported in Table S2, example 1, in the supplementary material.

The results obtained by analysing the doxorubicin liposomal formulation are summarized in Fig. 2. MD-AF4 shows a rather monodisperse sample, characterized by one single population of spherical particles with a hydrodynamic diameter of 72-78 nm (in good agreement with results obtained with batch mode DLS and cryo-TEM) and a shape factor (Rg/Rh) of about 0.8, which is typical of spherical liposomes internally carrying a crystallized API [17].

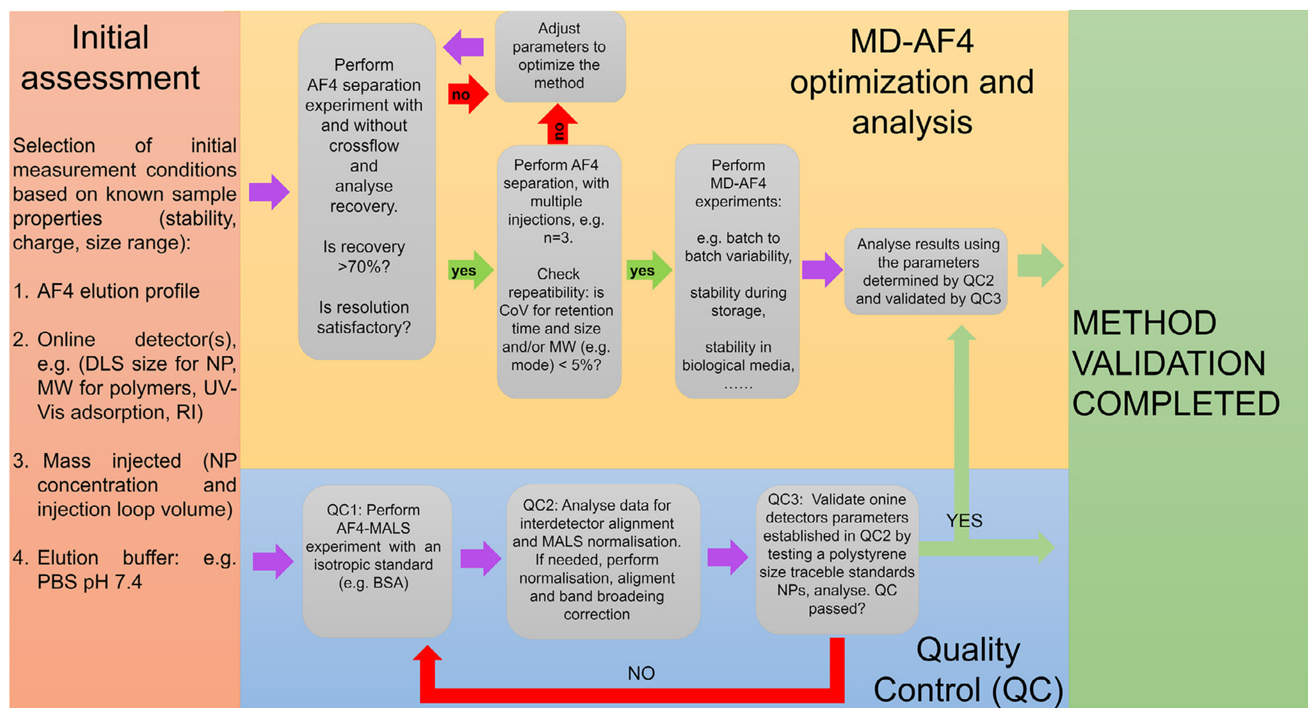


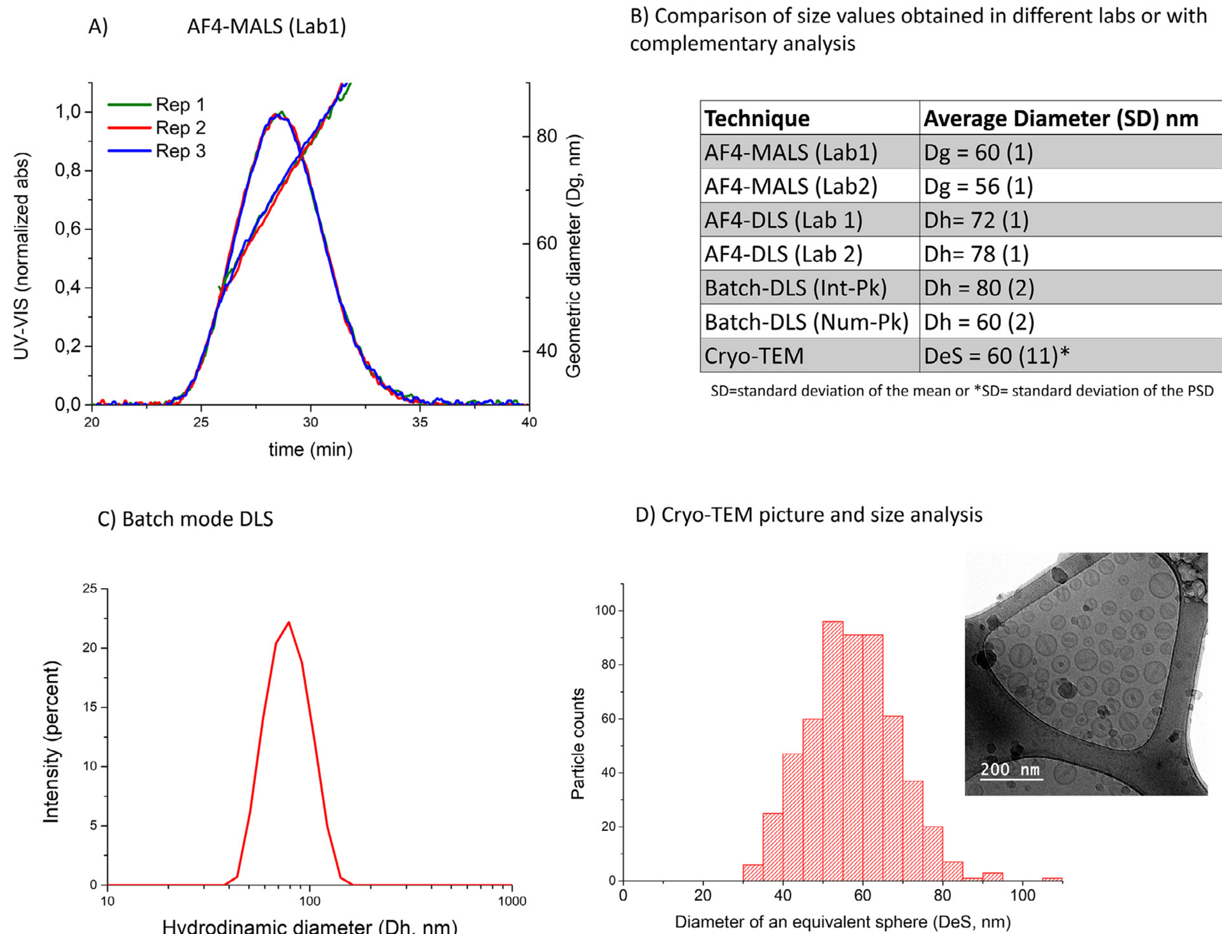
Fig. 1. Brief outline of the workflow of MD-AF4 measurements. Fours step are represented: 1) assessment of sample properties that helps determining the initial testing conditions (orange box); 2) MD-AF4 method optimization and testing according to ISO/TS 21362 (yellow box); 3) quality control to be run in parallel to MD-AF4 method optimization (blue box); 4) method validation completed (green box). Violet arrows represent data input, red arrows negative results, while green arrows successful experiments with results allowing stepping forward in the series of experiments. NP= Nanoparticle, BSA= Bovine serum albumin, QC= Quality control. CoV= coefficient of variation, PBS= phosphate buffer saline, MALS= multi angle light scattering, RI= refractive index.

The repeatability (three repeats between the same lab) and the reproducibility of the results obtained across different EUNCL laboratories equipped with different instrumental setup, as part of a preliminary ILC studies, are compared in Figs. 2A and 2B. Repeatability of the measurements in each AF4 setup was very satisfactory, with a coefficient of variation (COV) of the repeatability for each lab was $<$ 2.5% for the retention time of the peak (UV-VIS detector) and for the measurement of the average size by online MALS and online DLS (Rg and Rh respectively). However, small but significant differences in the size measurement results generated by two laboratories were observed, due to the intrinsic differences of the sizing detectors used (COV of around 5%). This very small ILC was the starting point for a larger collaborative ILC involving experts from NIST based on the NCI-NCL/EUNCL SOP and in light of the criteria reported by the ISO/TS 21362. The results of this ILC exercise, recently published [17], confirmed: i) the great robustness of MD-AF4 for measuring the physical properties of multiple liposomal formulation, and ii) small variations in the measurement of particle size can be generated by differences in the sizing detector configurations and by the software for data analysis provided by the different manufacturers. Nevertheless, for monodisperse formulation reproducibility assessment between different platforms showed that a coefficient of variation below 5% could be obtained. This is quite remarkable and demonstrate the potential of MD-AF4 for the physical analysis of liposomal formulations during formulation development. Furthermore, it shows the potential fro use of MD-AF4 for quality control purposes in pharmaceutical settings. The optimized protocol developed in this work is now the basis for a new ASTM standard test method focalized on the "*Physical characterization of liposomal drug formulations using multi-detector asymmetrical-flow field flow fractionation*" that is currently under consideration within the ASTM E56.02 committee. Importantly, this work is one of the first examples on how the collaboration between the EUNCL and the NCI-NCL infrastructure with regulatory bodies (such as EMA and FDA) and metrology institutes (such as NIST), led to a joint effort to develop a standard test method for addressing regulatory needs. Further ongoing efforts have been initiated, subsequently to this first experience on particle sizing, focalizing on the development of methods (i) for the chemical analysis (e.g. lipid composition of liposomes), (ii) for drug loading and drug release and (iii) for the *in vitro* safety assessment [10,32].

3. MD-AF4 is applicable for measuring a wide range of physical-chemical properties of nanopharmaceuticals

3.1. Size and morphology of a bimodal liposomal formulation

For the characterisation of monodispersed liposomal samples like Doxil, most of the reading audience would conclude that batch-mode DLS analysis could be sufficient to measure the average size and the particle size distribution (PSD) of the sample. The use of high-resolution techniques like MD-AF4 and TEM, indeed, provide additional confirmation but may be an excessive use of resources. However, as already shown by many studies [10,11,13,23,33], this is not always the case, and surprising results could be hidden even in the case of samples showing a single DLS peak with a Polydispersity Index (PdI) smaller than 0.2. An example of a more heterogeneous liposomal sample, where only measuring batch-DLS would have resulted in drawing the wrong conclusions is shown in Fig. 3. The batch-DLS analysis of these clinical-grade liposomes loaded with a topoisomerase I inhibitor shows only one population with a size of around 110 nm and a polydispersity index (PdI) $<$ 0.05, which is usually indicative of well monodispersed liposomes (Fig. 3). Surprisingly, the measurement



Technique	Average Diameter (SD) nm
AF4-MALS (Lab1)	Dg = 60 (1)
AF4-MALS (Lab2)	Dg = 56 (1)
AF4-DLS (Lab 1)	Dh= 72 (1)
AF4-DLS (Lab 2)	Dh= 78 (1)
Batch-DLS (Int-Pk)	Dh = 80 (2)
Batch-DLS (Num-Pk)	Dh = 60 (2)
Cryo-TEM	DeS = 60 (11)*

SD=standard deviation of the mean or *SD= standard deviation of the PSD

Fig. 2. Transfer of the SOP from NCI-NCL (Lab3) to EUNCL (Lab1, Lab2). A) The fractogram (repeatability) and the Dg measured by Lab1 is shown as an example. B) Comparison of average size values and standard deviation of the mean obtained by AF4 analysis (geometric diameter, Dg and hydrodynamic diameter, Dh) in the three labs, and size values obtained by orthogonal analysis of the same batch, including batch-mode DLS (Int-Pk=Intensity based distribution, Num-Pk= Number based distribution), and cryo-TEM are reported; C) intensity based PSD by batch mode DLS and D) cryo-TEM analysis. In the histogram and in the table the diameter of an equivalent sphere calculated by cryo-TEM is shown. Cryo-TEM was performed on only one sample, so the SD of the mean is not available.

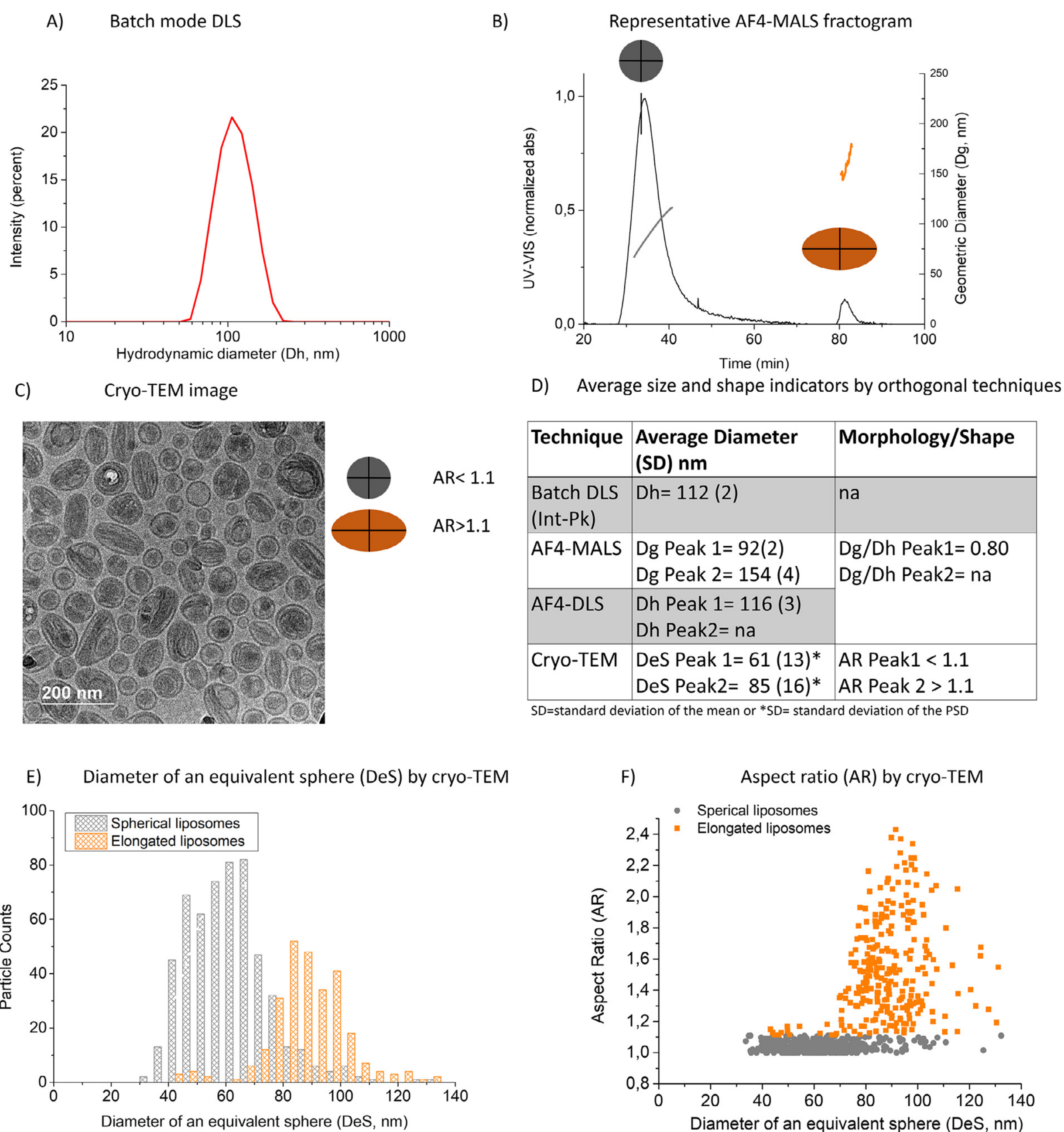
of this sample by AF4-MALS (experimental details in supplementary material, Table S2 - example 2) and by cryo-TEM show a more complex situation, demonstrating that this is a rather polydisperse sample composed of two distinct populations of liposomes, where batch-DLS was simply not able to resolve. The cryo-TEM analysis indicates that the two populations are characterized by two different shapes. The smaller population is composed of spherical vesicles possessing an aspect ratio (AR) smaller than 1.1 (population 1 represented by grey circles in Fig. 3C), while the larger population is characterized by elongated particles similar to coffee beans, with AR in the 1.1-4.0 range (population 2 represented by orange ellipses in Fig. 3C). In both populations it is possible to see the crystal of the drug inside the particles. AF4 could be also used to separate the various shapes, and collect fractions for further offline analysis like EM [11], chemical analysis, drug loading or *in vitro* assays to discriminate the potential role of different PCC properties, such as shape in determining biological effects. Calculating the shape factor (Dg/Dh) could also give information about the particle shape and morphology. As shown in Fig. 3D, the shape factor of 0.8 for peak 1 is consistent with spherical liposomes encapsulating API. Unfortunately, it was not possible to obtain the same information for the second population because the obtained values of hydrodynamic diameter by online DLS for non-spherical particles larger than 200 nm become unreliable. Similar examples on the use of MD-AF4 to identify multiple populations in apparently simple samples has been recently published by Hu et al. [11].

3.2. Drug release: measurement of the (in)stability in biological media

Most nanomedicine formulations are administered by intravenous injection, and for this reason their stability should be analysed in conditions representative of the physiological administration, such as, for example, in human serum [13,34,35]. The measurement of physical-chemical parameters such as size and drug loading and release in complex protein matrices (as human serum that contains a complex mixture of different proteins and components) is a difficult and time-consuming process, that requires the combination of different high-resolution techniques and careful protocol development.

MD-AF4 for measuring physical stability in plasma

For the analysis of size changes in presence of plasma proteins, MD-AF4 is one of the best techniques of choice. In fact, thanks to the addition of a separation step between particles and proteins prior to sizing detection, size can be measured at high resolution eliminating interferences generated by the free protein fraction. This is extensively described in the advanced MD-AF4 SOP [26]. Based on this, very promising results have been published by NCI-NCL or EUNCL on multiple samples, including liposomal formulations characterised by different PEG coverage [11] and lipid-based nanoparticles [9]. Furthermore, the recent work by Hu et al. also showed how AF4-MALS-DLS analysis could be used to qualitatively assess the amount of protein binding and amount of the interac-



Technique	Average Diameter (SD) nm	Morphology/Shape
Batch DLS (Int-Pk)	Dh= 112 (2)	na
AF4-MALS	Dg Peak 1= 92(2) Dg Peak 2= 154 (4)	Dg/Dh Peak1= 0.80 Dg/Dh Peak2= na
AF4-DLS	Dh Peak 1= 116 (3) Dh Peak2= na	
Cryo-TEM	DeS Peak 1= 61 (13)* DeS Peak2= 85 (16)*	AR Peak1 < 1.1 AR Peak 2 > 1.1

Fig. 3. Bimodal liposomes with particles possessing different size and shapes. a) Batch DLS, intensity-based size distribution of liposomes at 100 x dilution in PBS. Averages of 10 measurements. b) AF4 fractogram reporting the UV-VIS absorbance (black) and the geometrical diameters (Dg) determined by MALS of the two peaks (grey and orange). c) Representative cryo-TEM image. (d) Table summarizing the size values measured by different techniques. e) Number based size distributions by cryo-TEM. f) Size vs aspect ratio by cryo-TEM analysis. The diameter of an equivalent sphere is reported as size parameter for both populations.

tion of nanoparticles with plasma proteins by varying the PEG coverage on the surface of the liposomes [11].

MD-AF4 for qualitative information about burst drug release in complex media

In addition to changes in size distribution, another very important attribute to monitor in plasma is the drug release profile generated by the interaction of nanoparticles with proteins. The determination of the free *vs* encapsulated drug ratio and the measurement of the drug release kinetic profile in plasma are usually performed by combining an ultrafiltration step to separate the free drug from liposome-bound fractions, followed by HPLC-UV or LC-MS detection of the drug in the different fractions. As previously described [23], and also referenced by FDA [6], a careful evaluation of API lost due to possible interaction with the ultrafiltration device should be considered. For example, to ensure that the filtration step does not cause loss of material(s) [23]. As shown in the supporting Figure S1, in the case of doxorubicin, nonspecific binding of doxorubicin dispersed was observed when using filters made of regenerated cellulose for ultrafiltration prior to measurement of free *vs* encapsulated drug loading by LC-MS/MS. Saturation of the filters with the free API was necessary, prior to carry out reliable separation of the particles from the free drug components. The non-equilibrium filter binding observed significantly complicates the calculation of free drug fraction in simple media and in plasma when combining ultrafiltration and HPLC-UV-VIS or LC-MS/MS measurements. This should be taken into account with proper quality control in order to not bias the results [23]. Alternatively, the adsorption of the drug at various concentrations could

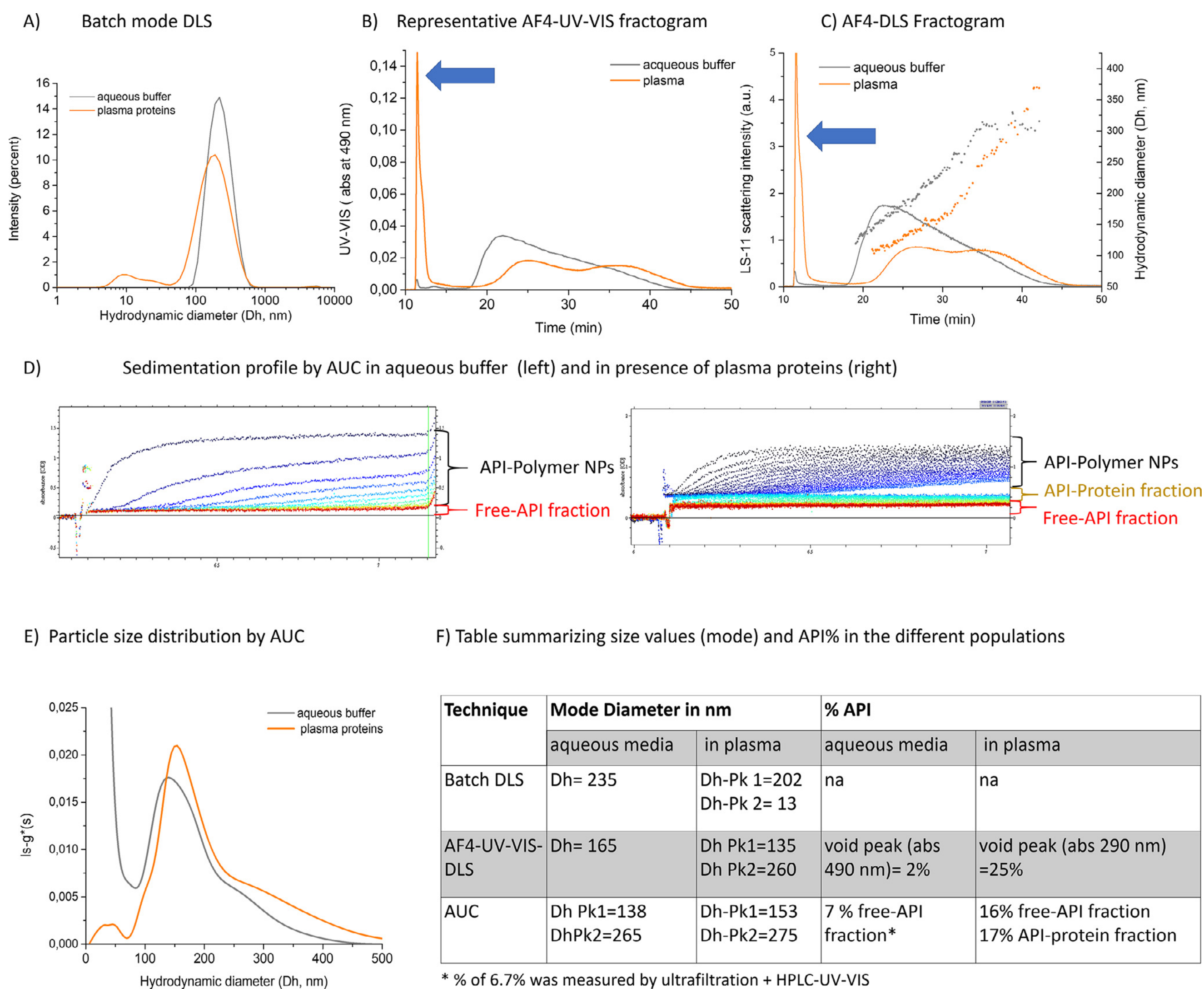
be monitored and corrected. A robust approach taking into account the API loss during ultrafiltration, is monitoring the presence, in the different fractions collected by ultracentrifugation, of a known concentration of a stable isotope tracer of the API, that is behaving as the API in the complex media, but that could be discriminated from the API in the MS profile [36–38]. This approach also allows to precisely measure the degree of API bonded to plasma protein, in addition to the free, and encapsulated API fractions. Despite being a very powerful method for the measurement of drug release in plasma, the *ultrafiltration plus MS detection* analysis using a stable isotope tracer is very expensive and time consuming. It is therefore suggested as the golden standard for the analysis of drug release in complex media, after performing a first screening of the formulation stability in plasma with less expensive techniques, such as MD-AF4 or analytical ultracentrifugation.

Here, we want to show that in some specific cases MD-AF4 can be used to identify a quick burst release of API in the presence of plasma proteins. Using MD-AF4 for such measurements does not require an ultrafiltration step prior to the analysis. It is important to remember, that this approach is only possible when drugs possess an absorbance at specific wavelength (e.g. doxorubicin at 490 nm, curcumin at 425 nm) where the structural components of the nanoparticle carrier (lipids, polymers) do not absorb/interfere. The experiment presented here was performed on a polymeric nanoparticle system conjugated to doxorubicin, named here as Doxo-Polymer conjugate, in order to distinguish it from the liposomal formulations presented earlier. The Doxo-Polymer conjugate was analysed by MD-AF4 (experimental details in supplementary material, Table S2 - example 3) in a buffered aqueous media and after incubation for 1 hour at 37°C with 10% commercial fetal bovine serum (FBS) in the same buffered aqueous media (see material and method sections in the supplementary materials for more details). As shown in Fig. 4A-4C, DLS and MD-AF4 analysis before and after incubation with FBS were able to confirm that the particle physical stability in serum was not impacted by particle interactions with serum proteins, e.g. no sign of major aggregation dramatically changing the particle size distribution profile. The most interesting evidence in the MD-AF4 measurement is the appearance of a well visible void peak detected with the UV-VIS detection at 490 nm, a wavelength where only the doxorubicin, but not the proteins or the liposomal excipients could absorb, and thus generate a signal (blue arrow in Fig. 4B). This peak is about 27% of the total integrated signal intensity of the fractogram, while in absence of serum it was only 2%, indicating that a significant amount of the drug is now eluted in the void peak, and thus have been released from the particles. It is important to remember that the doxorubicin drug in presence of plasma could be in three fractions: (i) in its free form outside the carrier (free-API fraction), (ii) bound to serum proteins outside the carrier (API-protein fraction) and (iii) encapsulated in the particles (API-polymer NP fraction). The free-API fraction is very small and it is probably not fully retained during focusing (doxorubicin MW=543.5 g•mol^{-1}) thus it may not be completely detected during the analysis, since a significant portion may go to the waste instead of reaching the UV-VIS detector. On the other hand, the doxorubicin bound to serum proteins will be eluted and registered by the UV-VIS detector in the void peak. In our specific case, using a gentle crossflow (the same used for analysing the particles), the doxorubicin-protein fraction appears in the void peak. Therefore, even if with MD-AF4 we cannot reliably quantify the total amount of the released drug, by seeing such an increase of the void peak we have an immediate indication of a burst drug release and of sample instability after incubation with plasma proteins.

In order to confirm the above results by a complementary technique, analytical ultracentrifugation was used to quantify the free *vs* encapsulated fraction in aqueous buffer and after incubation with plasma, applying the approach recently published by Mehn and al. [35,36]. Analytical ultracentrifugation (AUC) is a first principle-based technique, requiring no calibration by particle size standards. Particle size of polymeric nanoparticles and liposomes can be determined from their sedimentation speed in an aqueous medium. AUC can measure sedimentation of a nanopharmaceutical suspension using both absorbance and/or refractive index (RI) detector(s). If the density of the particles is known, the measured sedimentation coefficient distributions can be converted to mass-based size distributions. Moreover, as the molecular mass of a typical small drug molecule is generally about 5 orders of magnitude lower than the mass of an encapsulating nanoparticle, sedimentation speed of the free API is negligible compared to the sedimentation speed of the nanoparticles. If the API molecule has a specific UV-VIS absorbance, this results in a constant absorbance. The ratio of this "background" signal relative to the signal from the sedimenting nanoparticle fraction(s) gives information about the amount of free API outside the nanocarrier. Fig. 3B shows the sedimentation profile (the first, every fifth and the last measurement) detected at 490 nm for the sample before and after incubation with serum. In the aqueous buffer, the remaining stable background signal (the doxorubicin outside of the particles) correspond to 7.0± 0.3% of the total signal. This result is in very good agreement with HPLC-UV-VIS and LC-MS/MS measurements, where the amount of free drug in the formulation is calculated to be 6.7% of the total drug. AUC also provides an orthogonal confirmation of the PSD of the sample (Fig. 4E). Fig. 4D (right panel) shows the sedimentation profile of this sample measured at 490 nm after incubation with serum proteins. The data shows at least three different main species absorbing at 490 nm. First, at lower speed, the doxorubicin loaded particles (doxorubicin-polymer conjugates) are settling, followed by the sedimentation of the protein fraction bound to doxorubicin (API-proteins which is 16% of the total absorbance signal) at higher speed. Finally, the background absorbance corresponds to doxorubicin that does not sediment at 40000 rpm (free-API) and is about 17% of the total signal. Therefore, by AUC, we were able to confirm a burst release in serum of about 33% (the free API plus the protein-bound components) of the doxorubicin drug. This result is consistent with the amount of 27% obtained by MD-AF4. The discrepancy could be due to MD-AF4 underestimating the free drug fraction, that is not retained by the semipermeable membrane.

To further confirm the presence of unbound drug, we also assessed *in vitro* the cytotoxicity of the Doxo-Polymer conjugate by using two methods: the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction and the lactate dehydrogenase (LDH) release according to the EU-NCL-GTA01 and EU-NCL-GTA02 SOPs (available at www.euncl.eu/about-us/assay-cascade). As reference, free doxorubicin was included in the study. Results are presented in Figure S2 (LDH) and Figure S3 (MTT) and show at the highest time points of 24 and 48 h for both cell lines used (LL-CPK1 and HepG2) an evident dose-dependent toxicity in terms of LDH release as well as of reduction of the cell metabolic activity. The kinetic of toxicity induced by the Doxo-Polymer conjugate is similar to the one of the free| doxorubicin, indicating that a significant amount to the toxic API (doxorubicin) is quickly released from the nanoformulation. The data obtained were also in line with the morphological observation (data not shown).

To summarize MD-AF4 can be considered as a pre-screening technique to quickly identify stability issues of nanomedicine formulations in plasma, before considering the more expensive and time-consuming ultrafiltration and MS detection analysis using a stable isotope tracer. In cases like the one presented here, MD-AF4 and/or AUC combined with *in vitro* cytotoxicity measurements would be sufficient to detect problems of the formulation (in this



Technique	Mode Diameter in nm		% API	
	aqueous media	in plasma	aqueous media	in plasma
Batch DLS	Dh= 235	Dh-Pk 1=202 Dh-Pk 2= 13	na	na
AF4-UV-VIS-DLS	Dh= 165	Dh Pk1=135 Dh Pk2=260	void peak (abs 490 nm)= 2%	void peak (abs 290 nm) =25%
AUC	Dh Pk1=138 DhPk2=265	Dh-Pk1=153 Dh-Pk2=275	7 % free-API fraction*	16% free-API fraction 17% API-protein fraction

* % of 6.7% was measured by ultrafiltration + HPLC-UV-VIS

Fig. 4. Analysis of API-Polymer NP conjugate in aqueous buffer and after incubation with plasma proteins. A) PSD by intensity by batch DLS. B) UV-VIS fractrogram (detected at 490 nm) vs time. C) Light-scattering intensity and Dh vs time with and without plasma proteins. The blue arrow indicates the increase of the void peak in presence of plasma proteins. D) Sedimentation profiles and E) PSD by AUC with and without plasma proteins. F) Summary table reporting the measured size and % of API fractions not encapsulated in the NP with and without plasma proteins.

case an unpredicted burst release of the free API) that would need an optimization step before going into further expensive characterisation and/or *in vivo* experiments.

3.3. Drug Partitioning measurements

Drug loading and release is associated to the drug localization inside the nanoparticle carrier. Information where the drug ends up can give insight as to where the drug is initially located within a nanoparticle. For example, drug loaded into liposomes can either be bilayer-loaded or contained within the aqueous interior cavity. If the drug is bilayer loaded, it is possible for the drug to partition to other phospholipid bilayer structures via bilayer exchange mechanism [37]. Precise drug loading quantification of different nanoparticle populations and study of the drug exchange mechanisms, can be performed off-line with HPLC, after fraction collection and isolation by AF4, another very useful application of AF4 for nanopharmaceutical analysis.

To test the MD-AF4 technique for this application, liposomes loaded with a single drug were incubated with multilamellar large vesicles (MLVs) and analysed. MLVs were chosen as model system because they can be separated and resolved from liposomes by AF4 thanks to their different size, and also because they represent an interesting model of red blood cells, to simulate drug partition effects between nanoparticles and blood cells once the nanopharmaceuticals reach the blood vessels.

The MD-AF4 fractogram (experimental details in supplementary material, Table S2 - example 4) for the liposomes loaded with a single drug is shown in Fig. 5A; this serves as the control. The total drug recovered measured offline by HPLC was 88% across the three peak fractions collected. Of this, 92% was contained in the main peak with a size range of 75–128 nm. The second collected peak (71 - 88 minutes) corresponds to the time when the cross-flow rate is reduced to zero and represents any particles that were forcibly (artificially) agglomerated. In this case, 7.4% of the total drug recovered is present in this fraction. A very small amount, 0.6%, of drug was found in the sample loop cleaning step. The fractogram for the MLVs alone is shown in Fig. 5B. As expected, they elute at a much later elution time, namely when the cross-flow rate is reduced to zero. The MLVs ranged from 163–390 nm in size and contained no drug (as expected). A very small peak was observed during the sample loop cleaning step.

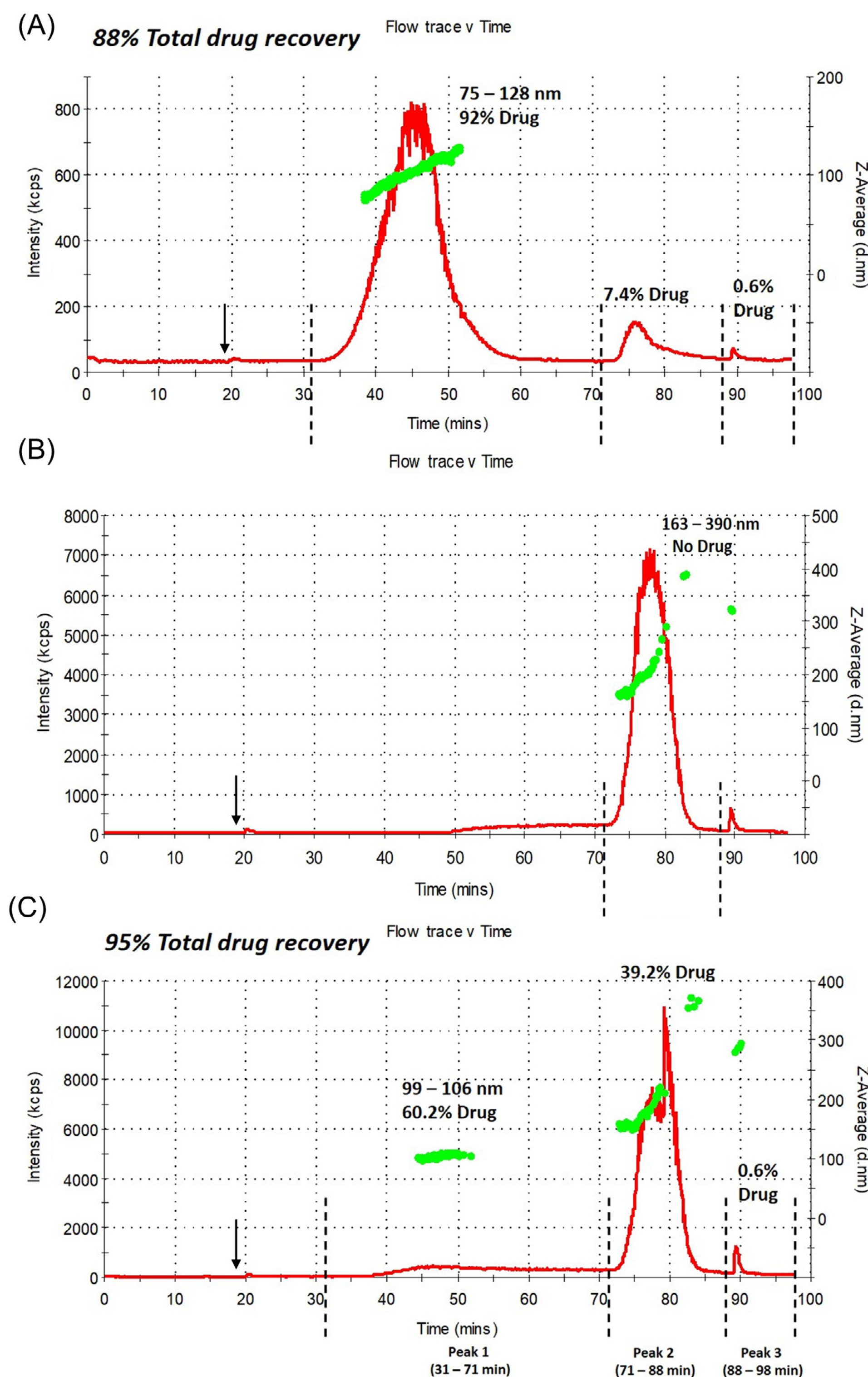


Fig. 5. Flow-mode diameter (AF4 separation with in-line DLS) for A) 'bilayer-loaded drug liposomes'. B) Multi-lamellar vesicles (MLV). C) 'Bilayer-loaded drug liposomes' incubated with MLV. The hydrodynamic size was measured across the peaks (green squares) using the on-line Malvern Zetasizer and was based on an intensity threshold of >500 (A), >500 (B), and >400 (C) kcps. The size ranges are given in the figure for each peak. Collected peak fractions are designated by dashed vertical lines along with the elution times. The % drug relative to the total amount recovered is given for each peak. Equilibration time (19') is indicated by black arrows.

The liposomes and the MLVs were co-incubated at 37°C for 35 minutes to test potential drug partitioning and their fractogram, as shown in Fig. 5C. Sample concentrations and conditions were identical to the individual runs (Figs. 5A and 5B). The same three peak fractions (i.e., elution times) were collected as in Fig. 5A and accounted for 95% of the total drug injected, representing a very good recovery. The first eluting peak, which corresponds to the liposomes, had a size range of 99 - 106 nm, and contained 60.2% of the total recovered drug. In comparison to the liposomes control (Fig. 5A), the drug distribution dropped from 92% to 60% af-

ter incubation with MLVs. The second peak, corresponding to the MLVs, had a similar size range as the MLVs alone and contained 39.2% of the total drug recovered. Note, the light scattering signal decrease within this peak is most likely due to multiple scattering (i.e. concentration is too high). Also, the liposomes peak is small due to its low concentration relative to the MLVs (compare Intensity in Figs. 5A and 5B). The last peak, representing the sample loop cleaning step, contained 0.6% of the drug, similar to the liposomes control (see Fig. 5A). Based on the HPLC measurement of the fraction associated to the liposomes peak before and after incubation with MLVs, a decrease of 31.8% of the drug content was observed in the liposomes, while the drug fraction associated to the MLVs peak increased to 39.2%. Interestingly, there is overlap between the second peak (71–88 minutes) in the liposomes control (see Fig. 5A) with the MLVs peak (see Fig. 5B). Accounting for this amount of drug, 7.4%, the drug transferred to the MLVs peak is 39.2%–7.4% = 31.8%, which is consistent to the decrease of drug in the liposomes peak. Hence, the data shows partitioning of the drug from one bilayer (single bilayer of liposomes) to another bilayer (multiple bilayers of MLVs). More importantly, the results demonstrate another application of the MD-AF4 analytical technique.

As mentioned earlier, the drug partitioning experiments can help to determine where the drug is initially loaded in the nanoparticle. However, it can also give insight as to the stability of the drug formulation in the presence of blood. Up to this point, partitioning of the drug to plasma proteins has been studied. Another important measurement is the drug partitioning into red blood cells as this will dictate its *in vivo* pharmacokinetic profile and biodistribution [37–39]. While this example did not use red blood cells, the MLVs could be used to represent them as an available lipid bilayer source to which the drug can partition. Although field flow fractionation of red blood cells [40–42] is not new, the use of MD-AF4 and MLVs to mimic red blood cells to determine drug distribution is novel. The MLVs provide a cleaner system and avoids all the complexities (i.e. decontamination of instruments, waste disposal, specialized training) associated with working with human red blood cells. Also, because of the large size of red blood cells, steric mode elution [43] may occur (large particles elute first) and the red blood cells might co-elute with the much smaller nanoparticles.

4. Conclusions and future perspectives

In this work, we have described the high-end use of MD-AF4 in the challenging field of characterization of nanomedicines. The joint effort, of the two state-of-the-art infrastructures for nanomedicine pre-clinical characterisation, namely NCI-NCI and EUNCL in developing SOPs and a standard test method has been a key factor in the successful application of MD-AF4 in this highly regulated field. On a more general level, it shows how the international cooperation, together with key interactions with regulatory agencies and metrological institutes, is helping the community by providing standard operating procedures and standard test methods that are so urgently needed to fill currently existing methodological gaps. Also, this kind of collaborations are hopefully contributing to the harmonization of the regulatory framework for nanomedicine characterization between Europe and USA.

In particular, MD-AF4 is a very versatile analytical technique to (i) measure size and shape distribution of highly heterogenous samples, (ii) to evaluate drug loading and drug partitioning, and (iii) to check particle (in)stability in plasma. Those are all key quality attributes to be monitored during formulation development and then after, for manufacturing quality control purposes. Size, shape, protein binding, and release kinetics can influence biodistribution, off-target toxicities, and ultimately safety and efficacy, while drug loading is essential for correct dosing. Interestingly, formulations that are unstable in physiological matrices, which prematurely aggregate or involuntary release drug(s), are unfit to meet the safety and efficacy criteria, thus risking to fail in their clinical translation. Importantly, the AF4 temporal/spatial fractionation prior to size analysis significantly enhances the analytical power *vs* many batch techniques, resulting in the high-resolution sizing analysis of complex heterogeneous samples or in plasma. Furthermore, when analysing drug loading and release, MD-AF4 can be used to rapidly and easily checking for burst drug release in plasma. MD-AF4 can thus fit as a very powerful technique for a "pre-screening" approach, giving semi quantitative indication of particle stability in plasma before proceeding with more expensive and complex analysis. Finally, fractions obtained by AF4 can be analysed off-line improving analytical resolution and identifying differences between different populations in a complex sample.

In conclusion, as presented in this review, MD-AF4 is a versatile technique for the physical-chemical characterisation of nanomedicines; either as single technique or when in combination with other complementary analytical techniques. It can be considered as an asset in the early preclinical R&D development settings, for getting accurate, robust and reproducible understanding of the physical properties and stability of potential nanopharmaceutics under pre-clinical evaluation. Similarly, during the later stages of product development and manufacturing, it could be one of the chosen techniques for quality control (e.g. batch to batch variability, burst release) within the pharmaceutical setting. Despite not being currently very widespread in the pharmaceutical industry, AF4 will become more widely used in the near future, due to continuous developments by the instrument manufacturers in line with industry needs (e.g. software meeting GLP requirements) and due to the availability of future newly developed standardized testing methods. Provided that the latter conditions will be met in a reasonable time, it is the view of the authors that MD-AF4 will become an enabling technology platform to provide high quality analytical characterisation supporting and enabling the regulatory evaluation of complex nanopharmaceutical formulations.

Authors' contributions and disclaimer

All the authors contributed to the writing of the article and approved the manuscript. MR and JC contributed to the EUNCL setting up and to the knowledge transfer. JC, FC, DM and LC contributed to the draft of the MD-AF4 SOP which is the base of the measurements described here. JC, FC, LC and DM performed the experiments reported in the case studies and performed the data analysis. SG performed the toxicological experiments and data analysis. FC, DM, JC, MR, APM, SEB, SG, and LC also provided critical discussion and revision to the manuscript.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461767.

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