Project:

Measuring NP Aggregation Propensities

Measuring the NP stability against aggregation as a function of time and buffers with batch-mode DLS

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1.0	20/02/2016	All	Initial Document	Luigi Calzolai

Table of Content

1 Introduction

Nanoparticles (NP) tend to lose their colloidal stability and aggregate if they are not properly stabilized. Generally speaking their aggregation propensity depends on temperature, time, pH and ionic strength of the dispersant/medium. These properties need to be checked in order to predict how NP will behave when performing in vitro (or *in vivo*) studies.

The stability of nanoparticles against aggregation can be assessed using batch-mode dynamic light scattering.

This document provides guiding principles for a quick assessment strategy that aims to maximize the relevance of the information obtained, while minimizing the measurements.

It is important to note that the strategy needs to be adapted to each particular NP system under study. In addition, the starting point should be the procedure and details provided by the sponsor.

2 Principle of the Method

For details on sample preparation for a DLS measurements, on how to perform a DLS measurements, and on the standards to be used to check the DLS equipment refer to the last version of the SOP on DLS (EU-NCL_PCC01:"Measuring Batch Mode DLS"). The aim of this protocol is to describe how to assess NP stability in different experimental conditions.

3 "Smart" strategy to evaluate stability against aggregation

The strategy consists of 6 steps:

- Optimization of the concentration range to be used in the protocol (step 1),
- measurement of NPs size in four different media (step 2), including MilliQ water, the buffer used for zeta potential measurements, 1x phosphate buffered saline (PBS) and 1x PBS+10%FBS,
- concentration series in 1x PBS (step 3)
- A kinetic in 1x PBS at 37°C (step 4)
- Study of the stability of the NP stock *vs.* time (step 5)
- Study NPs behavior at different pH (step 6, to be performed only for some particular samples) ,

Unless specified (see step 4), measurements are always done at 25°C. The details on how to perform each step are described below.

Step 1: Optimization of the concentration for DLS analysis

As a first step, the suitable concentration range to perform the protocol should be determined. Sample for DLS measurements should be neither too concentrated (e.g. to avoid multiple scattering effects) nor too diluted (to guarantee a suitable signal to noise ratio).

To perform this step a suitable buffer should be selected according to the NPs tested. Chose the buffer suggested by the sponsor or MilliQ water.

Start by a choosing reasonable concentration, according to the indications given by the sponsor. For organic NPs, the higher concentration to start with (see step 2) may be in the range of 1 mg/mL (lipid/polymeric total mass/mL). Do not take it as a general indication, since the optimum concentration for the measurement is very sample dependent and should be founded case by case. If needed, perform different measurements by changing the concentration to select an optimal concentration to perform step 2.

The optimal concentration range for the measurements depend on the sensitivity of the instruments. A concentration which guarantee a good signal to noise ratio, with no multiple scattering phenomena (see below how to check the quality of a DLS measurement) and which gives you with attenuator of 6 or 7 should be selected as a starting point for the following steps. However, with instruments possessing a very high sensitivity (Malvern ZSP), if comparability with other instruments is necessary (e.g. Malvern ZS), concentration with attenuator factor>4 is acceptable as starting point for a dilution series (provided that the conditions described above are satisfied).

The quality of DLS measurements should be always checked by looking at the autocorrelation function (see EU_NCL_PCC001) and at the value of the intercept of the autocorrelation function, which should be around 1 (>0.9). In reliable conditions (good concentration range), a series of repeated measurements of the same dispersions (e.g. 5- measurements of the same cuvette) should provide comparable z-average and PdI (no significant differences between the different replicas with cumulant analysis). Moreover, the derived count rate should be proportional to NP concentration (increased proportionally to NPs concentrations). If such conditions are satisfied, you have found a suitable concentration range for your DLS measurements.

Only for step 1, you can adopt the following SOP:

***Table 1*: SOP for DLS measurements, step 1**

Parameter	Value
Equilibration time	> 5' (1°C/min)
Scattering Angle	173°
Number of Measurements	5
Number of Runs	12
Run duration	10 s
Delay between measurements	0 s
Volume inside the cuvette	According to the manufacturer' instructions
Positioning Method	Seek for optimum position
Automatic attenuation selection	yes
Analysis mode (Malvern)	General Purpose

NB: Step 1is to be considered a preliminary step. Therefore, up to this point it may be not necessary to run a NIST traceable standard for quality control (QC) purposes. Moreover, to reduce the time

spent in step one, the number of measurements for each cuvette can be reduce to 5 (instead of 10 as in the rest of the protocol).

After step 1 has been completed the measurement of a NIST traceable standard should be performed for QC as described in the SOP EU_PCC_NCL001.

Step 2: Buffer effects at 25°C

Measure DLS according to the SOP on batch mode DLS of the samples in various buffer solutions at the starting concentration selected in step 1. The SOP to follow from step 2 on is reported in table two.

***Table 2*: SOP for DLS measurements to be used in step 2, 3, 5 and 6**

Parameter	Value
Equilibration time	> 5' (1°C/min)
Scattering Angle	173°
Number of Measurements	10
Number of Runs	12
Run duration	10 s
Delay between measurements	0 s
Volume inside the cuvette	According to the manufacturer's instructions
Positioning Method	Seek for optimum position
Automatic attenuation selection	yes
Analysis mode (Malvern)	General Purpose

Differently from step 1, you should perform 10 measurements for each condition tested:

- Buffer 1: MilliQ water or specific suitable buffer suggested by the sponsor used in step 1
- Buffer 2: Low saline buffer selected for zeta potential measurements: first choice will be 10 mM PB (NaH2PO4 4.23mM; NaH2PO4 5.77 mM) at pH 7.4. If aggregation of NPs occurs in this buffer, check NaCl 10 mM or PBS 0.1x to choose the right alternative for zeta potential measurements
- Buffer 3: 1x PBS at pH 7.4 or 154 mM NaCl at pH 7.4
- Buffer 4: 1xPBS +10% fetal bovine serum (FBS)

NB: After selecting the right buffer for zeta potential measurements, a zeta potential measurement should be performed as described in the SOP EU-NCL_PCC002.

Step 3: dilution series in PBS 1x

Perform a concentration series in PBS, by starting from the concentration used in step 2. From this concentration, perform at least a 10x dilution, a 50x dilution and a 100x dilution.

NB: If you perform serial dilutions from the dispersion prepared in step 2, check sample stability during the measurements. Serial dilution should be avoided if sample is not stable during the time of the measurements. Always prepare the sample by dilution of >10 µL to avoid errors in pipetting.

Step 4: Temperature effect in physiological buffer

Measure at 37 °C in Buffer 3 (PBS 1x) for 12 h (e.g. let the measurement run overnight) at 30 minutes intervals, using the concentration selected for "Buffer effect" in step 2. The following SOP should be used for this step:

***Table 3*: SOP for DLS measurements to be used in step 4**

Parameter	Value
Equilibration time	12 minutes
Scattering Angle	173°
Number of Measurements	25
Delay between measurements	1800 s
Number of Runs	12
Run duration	10 s
Volume inside the cuvette	According to the manufacture
Positioning Method	Seek for optimum position
Automatic attenuation selection	yes
Analysis mode (Malvern)	General Purpose

Step 5: Time dependency

Store NPs under conditions suggested by the Sponsor. If NPs are stored in liquid state, perform one DLS measurement in water or in a suitable buffer indicated by the sponsor at the concentration used in step 2. Time point should be chosen in agreement with sponsor and with the other analysis to be performed.

Step 6: pH effect (optional)

Titration vs. pH in MilliQ water at concentration with attenuator 6 or 7 may be performed for some samples. This measure is optional and depend on the way of administration/action of the NPs. If needed it can be associated to measurements of the zeta potential vs. pH.

4 Equipment and Reagents

4.1 Equipment

- ZetaSizer Nano ZS or similar equipment (Malvern Instruments)
- Disposable Cells

4.2 Reagents

- Buffer 1: MilliQ water or specific buffer indicated by the sponsor
- Buffer 2: 10mM PB buffer pH 7.4 (NaH2PO4 4.23mM; NaH2PO4 5.77 mM), or other condition used for zeta analysis, including 10 mM NaCl or PBS 0.1x

- Buffer 3: 1x PBS (NaCl 137 mM, KCl 2.7 mM, Na_2HPO4 10 mM, KH_2PO_4 1.8 mM)
- Buffer 4: 1x PBS + 10 % FBS

Refer to specific protocols for the preparation of the buffers.

4.3 Measurement Cells

Use a new disposable cell rinsed with the corresponding buffer for each measurement as indicated in the SOP EU-NCL_PCC_001_

4.4 Properties of the different buffers used in this protocol

The data analysis requires the knowledge of some properties of the system. In particular, the viscosity and refractive index of the liquid phase for the cumulants method. The estimation of the volume and number-based particle size distribution requires also the refractive index of the particles.

***Table 2*: properties of different aqueous media**

Aqueous Medium	Absolute viscosity (mPa . s)			Refractive Index
	20 °C	25 °C	37 °C	
pure water	1.002	0.890	0.692	1.332
10 mM NaCl	1.003	0.891	0.693	1.332
Phosphate buffered saline (PBS)	1.023	0.911	0.713	1.334
PB 10mM	1.002	0.890	0.692	1.332
154 mM NaCl	1.020	0.908	0.710	1.334

4.5 Additional measurements to be performed related to this protocol

4.5.1 To be performed before this protocol: UV-VIS spectra

- Before starting DLS measurements, an UV-VIS spectra of the sample should be recorded, as explained in the general SOP for DLS analysis, the EU-NCL-PCC001.

4.5.2 To be performed after this protocol: pH measurement

- From step 2 on, pH should be measured after each DLS measurement, as described in the dedicated SOP (See EU_NCL_PCC0x, Measurement of pH).

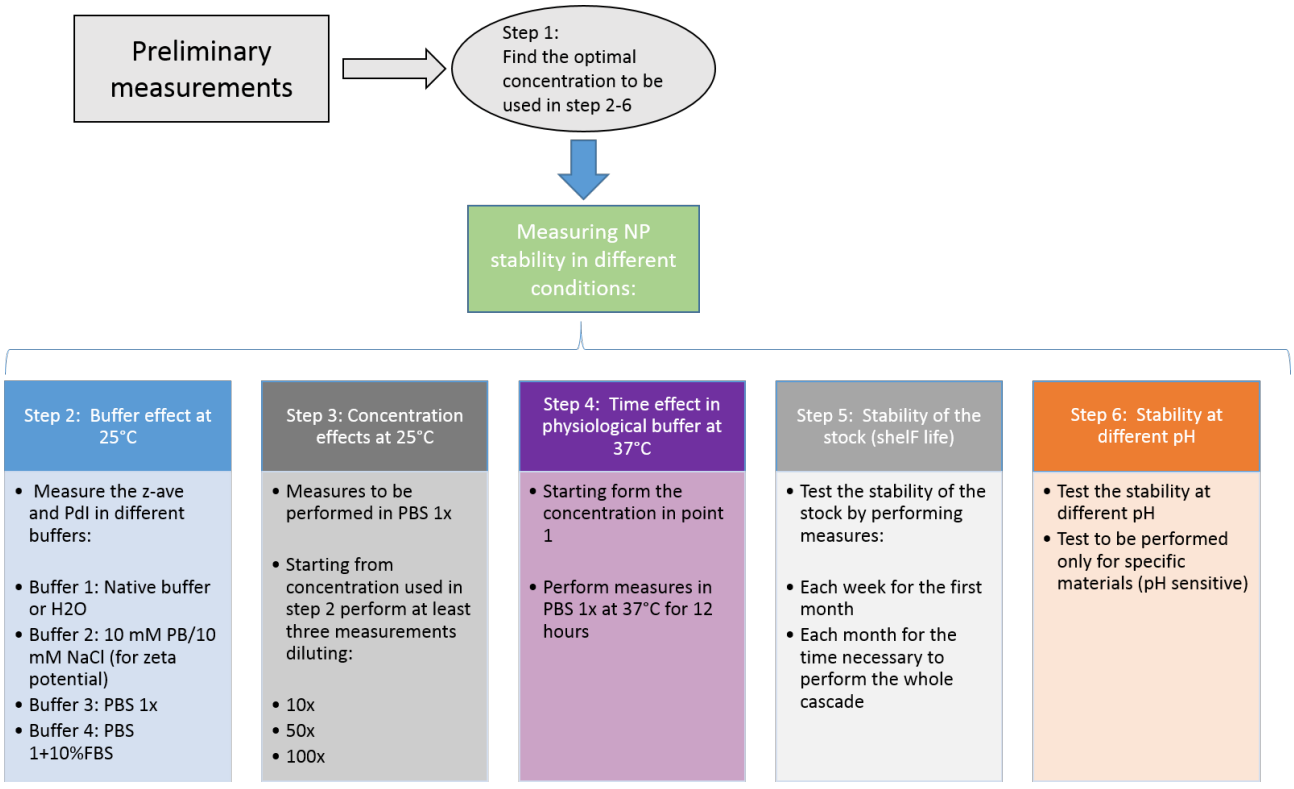
4.6 Measurement of NIST traceable standard for QC

A NIST traceable standard for size should be measured for QC purposes from step 2 on, as reported in details in the SOP EU_NCL_PCC001. Measurement of the standard should be performed at least once a day. If this protocol takes more than one day (from step 2 on), the measurement should be repeated at least once at the beginning of each day. The following SOP can be used for the measurement of a NIST traceable standard:

***Table 1*: SOP for DLS measurements, NIST traceable standard**

Parameter	Value
Equilibration time	> 5' (1°C/min)
Scattering Angle	173°
Number of Measurements	5
Number of Runs	12
Run duration	10 s
Delay between measurements	0 s
Volume inside the cuvette	According to the manufacture
Positioning Method	Seek for optimum position
Automatic attenuation selection	yes
Analysis mode (Malvern)	General Purpose

4.7 Flow chart of the protocol



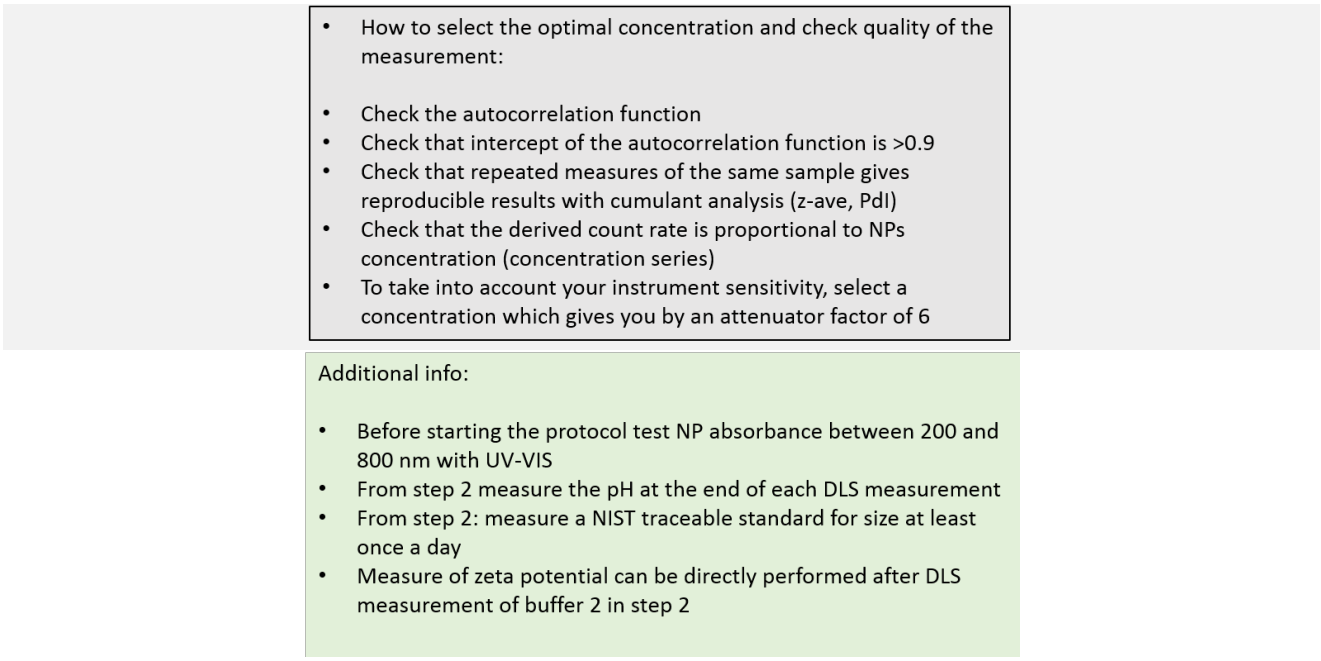


Figure 1: Summary of the protocol

5 Reporting data

Always report the name of the sample, the operator who performed the measurements, the protocol used to prepare the sample, the details of the SOPs and the details of the equipment used for the measurements.

An example of a report is reported below:

Experiment

Sample ID	Eu-NCL code
Date of experiment	15-19.10.2015
SOPs	EUNCL_PCC_01 v 0.9; EUNCL_PCC_013 v1.0
Partner	CEA
Operator	Fanny Caputo

Parameters

Particle concentration	Unknown (Total lipid: 17.4 mg/mL)
Sample preparation	100x dilution in filtered dispersion medium

	Filtration: 0.2 µm disposable syringe filter PTFE (Millex, Millipore)
Particles RI	1.46
Particles Absorption	0.01

Buffer properties

Dispesion medium	T	Viscosity	RI	Dielectric constant
H_2O MilliQ	25°C	0.890	1.332	78.6
10 mM PB, pH 7.4	25°C	0.890	1.332	78.6
10 mM NaCl	25°C	0.890	1.332	78.6
PBS 1x	25°C	0.911	1.334	78.6

Instrument details

Instrument	Malvern Zetasizer, NanoZSP
Wavelength	633 nm
Scattering angle	173°
Cuvette	Disposable PMMA semi micro cuvette 10 mm path

Measurement details

Equilibration time	300 s
Number of measurements	10
Number of runs	12
Run duration	10 s
Analysis mode	General purpose
File name	151015_control liposomes_633_stability

5.1 Buffer effects at 25°C

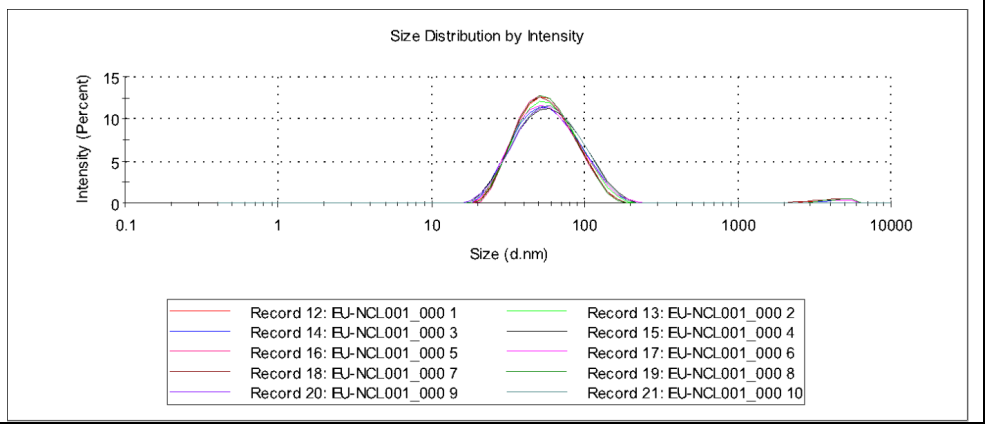
1) Report the details of the buffer series as in the following table:

Buffer	Attenuator	Derived count rate	Typical count rate
Buffer 1	x	71468,2	250
Buffer 2	x	85069,5	300
Buffer 3	X	84786,9	300
Buffer 4	X	82122,5	300

2) Report the results of cumulants analysis and PSD as in the following example:

Sample	Cumulants Method		PSD analysis		
XXXX 100x diluted	Z-average ± SD	PDI ± SD	intensity-PSD (mean) ± SD	σ of peak 1	% of peak area ± SD
Buffer 1 MilliQ	90.68± 0.43	0.045 ± 0.007	96.19 ± 0.46	25	100 ± 0 %
Buffer 2 PB 10 mM	81.67± 0.39	0.021 ± 0.012	85.5 ± 0.6	19.12	100 ± 0 %
Buffer 3 NaCl 10 mM	81.25± 0.49	0.026± 0.016	85.35± 0.73	20.32	100 ± 0 %
Buffer 4 PBS	80.92± 0.50	0.018 ± 0.016	84.6 ± 0.8	18.92	100 ± 0 %
Buffer 5 PBS + 10%FBS	73.03± 0.33	0.256 ± 0.009	95.85 ± 1.31	31.7	98.3 ± 2.7 %

3) Report the PSD plot of intensity distributions of all the conditions measured:

PSD Plot At concentration X in buffer Y	

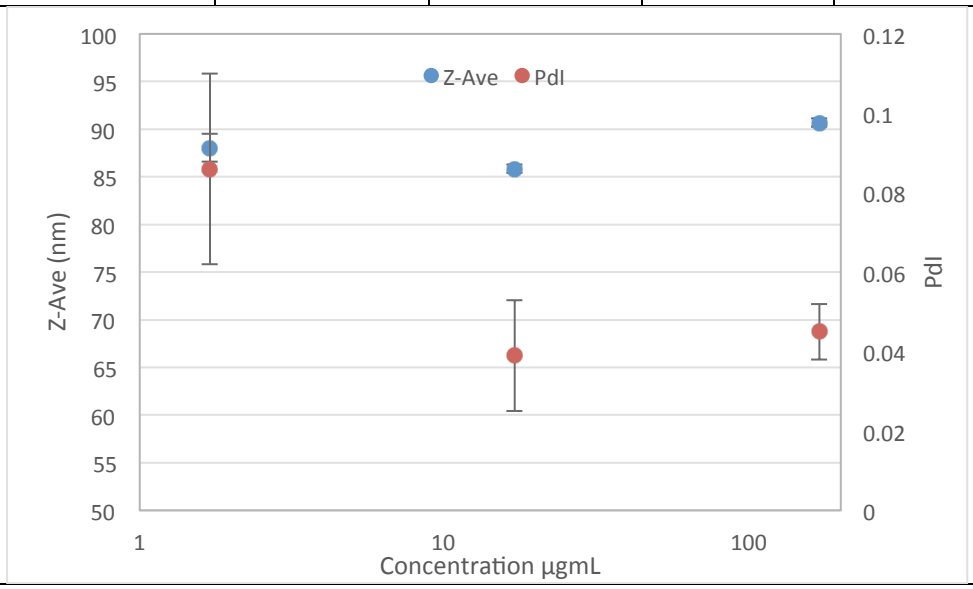
5.2 Concentration series:

1) Report the details of the concentration series:

NP concentration	Attenuator	Derived count rate	Typical count rate
10x dil[1] (170 µgmL)	6	71468,2	275
100x dil (17 µgmL)	8	6349,1	258
10x dil (1.7 µgmL)	10	730,4	204

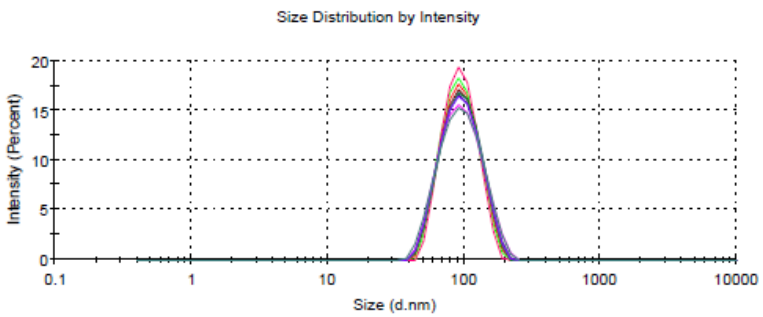
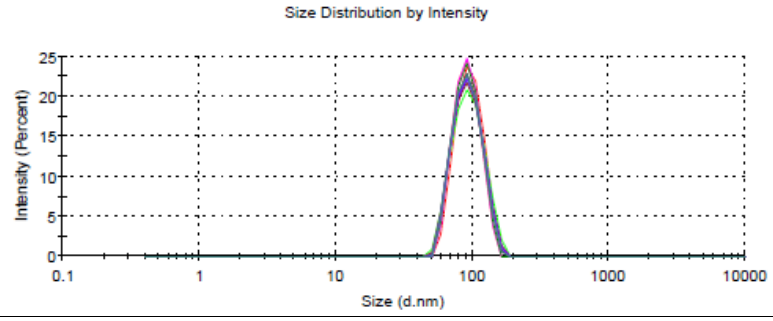
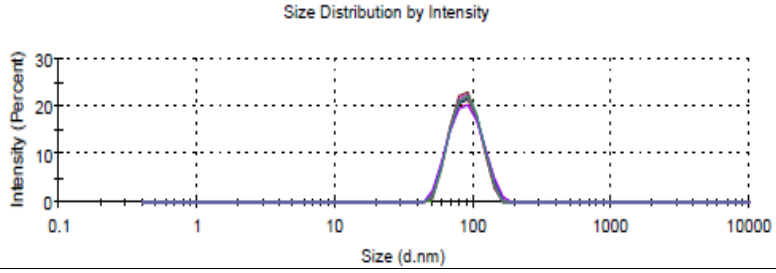
[1] The values refers tot he dilution from the stock, which is concentrated 17 mg/mL

2) Report the details of DLS results by cumulant analysis and PSD analysis:

Sample	Cumulants Method		PSD analysis		
	Z-average ± SD	PDI ± SD	intensity-PSD (mean) ± SD	σ of peak 1	% of peak area ± SD
10x dil[1] (170 µgmL)	90.68±0.43	0.045±0.07	96.19 ±0.96	24.03	100 ± 0 %
100x dil (17 µgmL)	85.86±0.45	0.039±0.014	90.91±0.67	25.22	100 ± 0 %
10x dil (1.7 µgmL)	88.05±.45	0.086±0.024	96.18 ±2.2	31.72	100 ± 0 %
Z-Ave and PdI					

[1] The values refers tot he dilution from the stock, which is concentrated 17 mg/mL

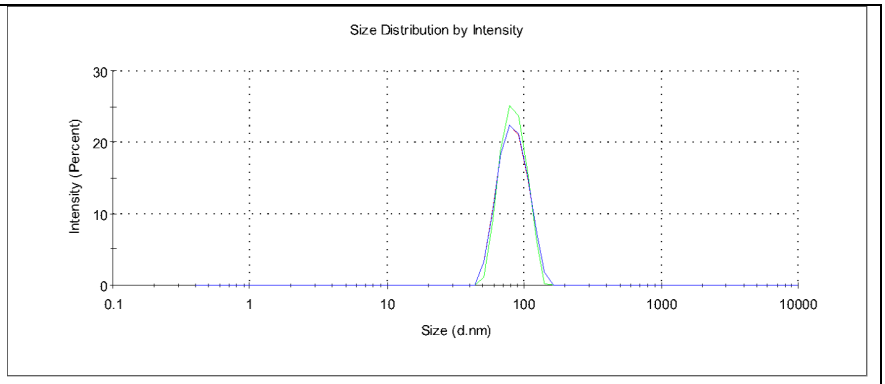
3) Report the hydrodynamic size distribution by intensity for each condition tested

PSD Plot 10x	
PSD Plot 100x	
PSD Plot 1000x	

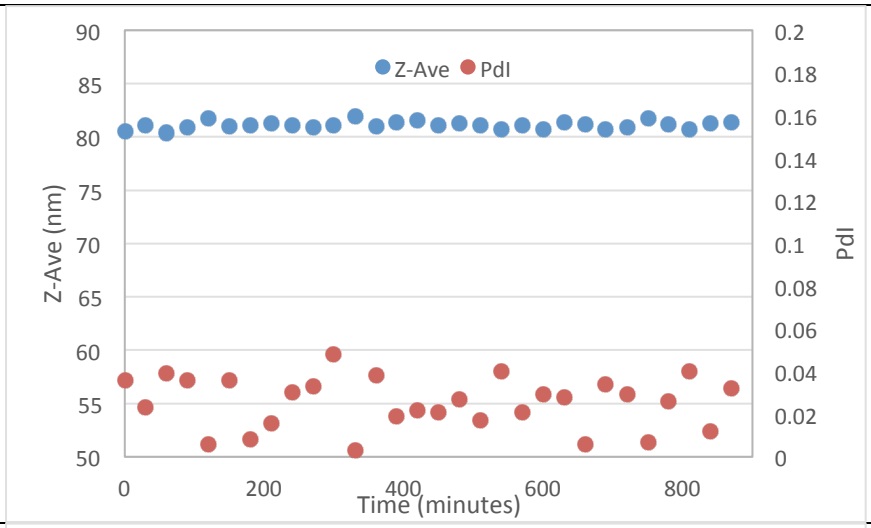
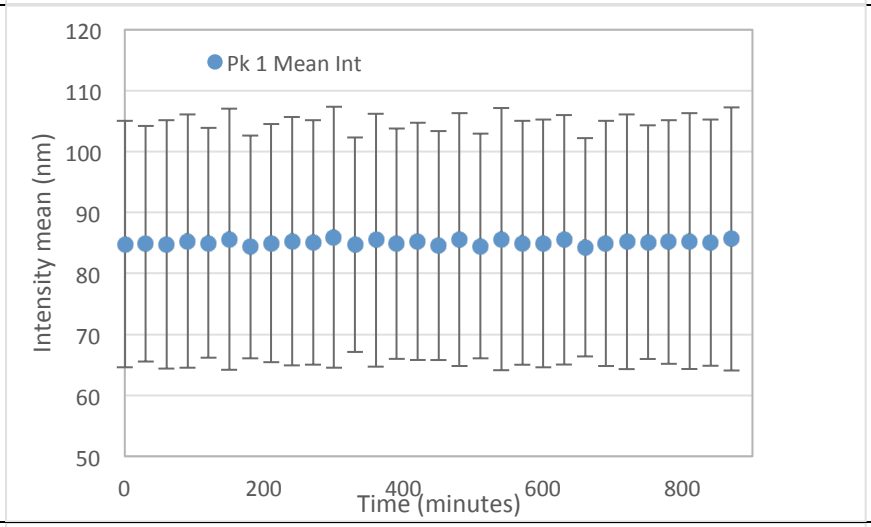
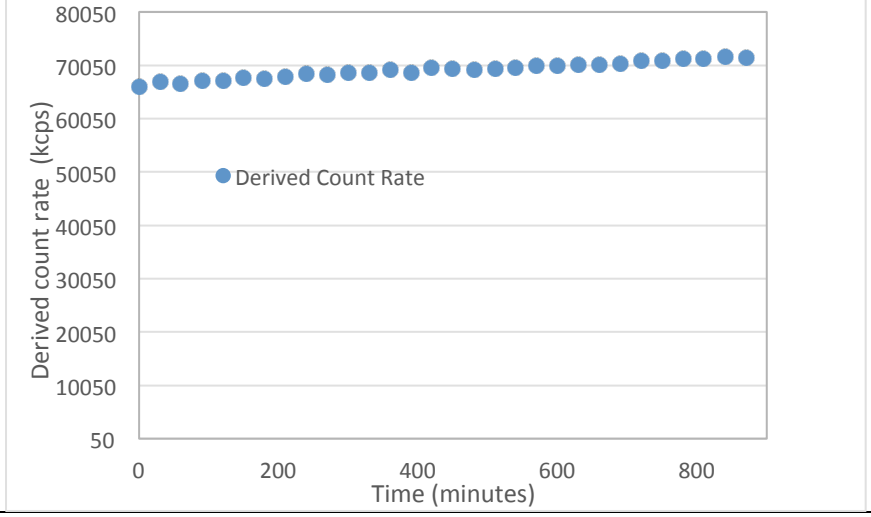
5.3 Temperature effect in physiological buffer

Report the DLS Results as in the following example:

Sample	Cumulants Method		PSD analysis		
DOX-NP CTRL 100 x diluted in PBS 37°C	Z-average ± SD	PDI ± SD	intensity-PSD (mean) ± SD	σ of peak 1	% of peak area ± SD
Average	81.09± 0.34	0.025 ± 0.012	85.1 ± 0.4	21.5	100 ± 0 %

PSD Plots At 0, 450, 720 min	

Time point (s)	Z-average	PDI	intensity-PSD (mean)	σ of peak 1	% of peak area
0	80,53	0,036	84,81	20,2	100
30	81,08	0,023	84,85	19,32	100
60	80,35	0,039	84,75	20,36	100
90	80,83	0,036	85,31	20,77	100
120	81,72	0,006	85	18,84	100
150	80,97	0,036	85,63	21,43	100
180	81,12	0,008	84,32	18,29	100
210	81,3	0,016	84,98	19,55	100
240	81,05	0,03	85,3	20,41	100
270	80,94	0,033	85,12	20,08	100
300	81,02	0,048	85,95	21,41	100
330	81,91	0,003	84,7	17,59	100
360	80,98	0,038	85,46	20,79	100
390	81,31	0,019		8 18,91	100
420	81,5	0,022	85,27	19,46	100
450	81,01	0,021	84,6	18,8	100
480	81,29	0,027	85,54	20,72	100
510	81,05	0,017	84,51	18,43	100
540	80,76	0,04	85,63	21,5	100
570	81,11	0,021	85,02	20,02	100
600	80,72	0,029	84,9	20,32	100
630	81,32	0,028	85,55	20,49	100
660	81,21	0,006	84,29	17,92	100
690	80,7	0,034	84,94	20,15	100
720	80,86	0,029	85,21	20,9	100
750	81,71	0,007	85,14	19,2	100
780	81,14	0,026	85,17	19,99	100
810	80,69	0,04	85,3	20,97	100
840	81,27	0,012	85,06	20,2	100
870	81,32	0,032	85,67	21,6	100

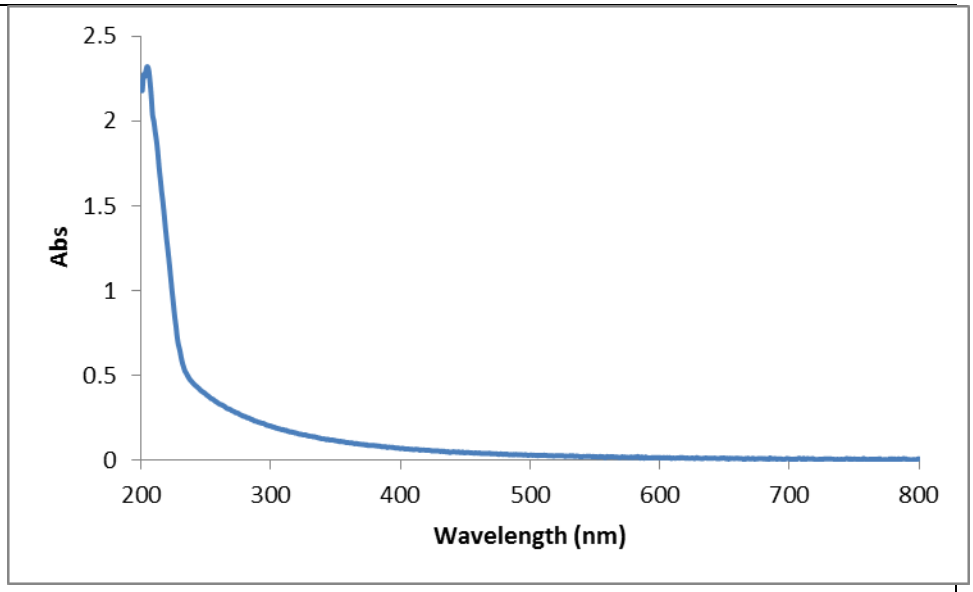
Z-average time dependence	
Intensity mean time dependence (error bars represent σ)	
Count rate stability	

NB. For the pH effect and stability of the stock follow the guide lines reported in the sessions above.

5.4 Supplementary information:

Always include in the report as supplementary information:

1. UV-VIS spectra

UV-VIS spectra	

2. The measurement of the size of a NIST traceable standard:

Reference of the measured standard	NIST traceable PLS 60 nm from Thermoscientific (ref n° XXX, lot n°)
Size and PdI by cumulant analysis:	x
z-ave and PdI according to the certificate of the standard±10%	x
System suitability test passed	Yes/No

NB: Validation of instrument performances with a standard should be always performed before starting the study. Instrument performances should be tested with traceable standards according to the QC procedure (See also EU-NCLPCC001).

3. The measurement of the pH of the samples and the information on the calibration of the electrode, as reported below:

pH after measurement	7.4

Calibration points pH	4, 7, 10
Electrode efficiency	98% of the theorical value

NB: pH measurement is accepted if electrode efficiency is above 95%