DRAFT Appendix to provide details on flash freezing of dispersed samples according to Nanogenotox dispersion SOP section 9.3

Johannes Keller, Daniel Quevedo & Wendel Wohlleben

BASF SE, Dept. Experimental Toxicology and Ecology, and Dept. Material Physics, 67056

Ludwigshafen, Germany

johannes-georg.keller@basf.com, wendel.wohlleben@basf.com

Scope:

The NANoREG Dispersion SOP, while acceptable for the dispersion of single materials for characterization, would be cumbersome in situations where timing is a factor. For example, in an experiment where many ENMs need to be tested for toxicity, there would be a time interval of around 20 minutes between each dispersion being ready. Even if only eight materials are dispersed, it would result in a time difference of 160 minutes between the dispersion of the first and last material. The SOP also calls for an immediate check of dispersion quality using DLS, which adds more time. As a result, fundamentally differently treated dispersions would be used in the experiment, which would potentially affect the resulting data. To resolve this bottleneck, pre-dispersed frozen suspensions are recommended by section 9.3 in the NANoREG dispersion SOP. The present SOP describes the procedure of flash freezing of stock solutions after dispersion according to (Vila et al. 2017), as mentioned shortly in section 9.3 in the NANoREG dispersion SOP

Dispersion

Before dispersion, the nanomaterial (NM) is prewetted in 0.5% absolute ethanol and afterwards water with 0.05% BSA content is added to achieve a concentration for the stock dispersion of 2.56 mg/mL. The solution is then dispersed with a fingertip sonicator for 16 minutes (Branson 550 probe sonifier with a 3mm tip, calibrated to an output power of 7.35 W).

Freezing

Up to five minutes after dispersion of the stock solution following the NANoREG SOP, the solution is aliquoted into 1.5 mL microcentrifuge tubes (Eppendorf, 0030 120.191) and flash frozen in liquid nitrogen (LN_2). The tubes were pre-prepared by puncturing the cap with a small hole to prevent decompression at low temperatures. The tubes are immersed in a dewar vessel filled with LN_2 for two minutes and then placed in long term storage at -80°C. The facilities at BASF do not have the standard -80°C freezers and thus we improvised by storing our samples in dry ice (-78.5°C). Cryo-protective gear, namely the use of gloves and long tweezers were used to protect the user, and all work was done in a chemical hood.

Thawing

After flash freezing, each aliquot was thawed as needed. This was done by placing each aliquot in a hot (~65°C) bath sonicator for 1 minute, or until a small ice core was left in the tube. Immediately following thawing, the ENM suspensions were either measured or diluted 10x in cell media (Dulbecco Modified Eagle Medium + 10% Fetal Bovine Serum + 1% Non-essential Amino Acids + 1% Pen/Strep) and incubated for 1 hour at 37°C. Sufficient sample was diluted for each intended application. After the incubation, the incubated samples were characterized using one of three techniques: DLS, VCM, or AUC as seen in ***Figure 1***.

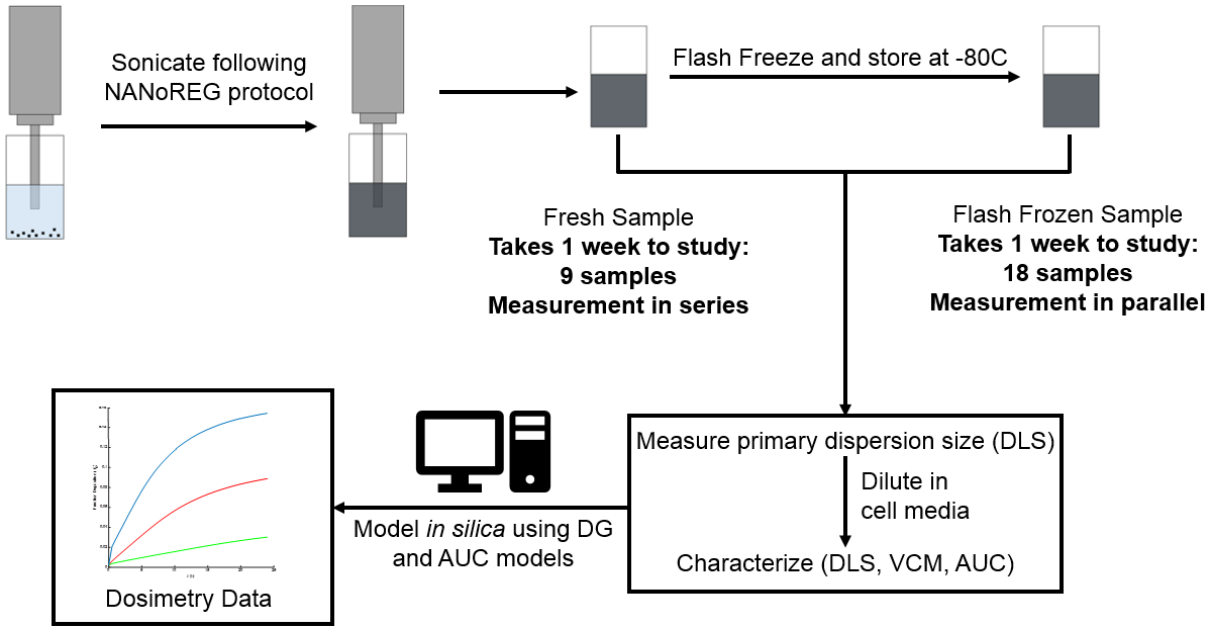

***Figure 1**: Schematic summary of workflow design.*

The original paper already checked size distribution, ion content and in vitro effects. Some of the reference materials are identical to PATROLS materials.

Vila et al (2017) abstract: *The variability observed in nanoparticle (NP) dispersions can affect the reliability of the results obtained in short-term tests, and mainly in long-term experiments. In addition, obtaining a good dispersion is time-consuming and acts as a bottleneck in the development of high-throughput screening methodologies. The freezing of different aliquots from a stock dispersion would overcome such limitations, but no studies have explored the impact of freezing thawing the samples on the physico-chemical and biological properties of the nanomaterial (NM). This work aims to compare fresh-prepared and frozen MWCNT, ZnO-, Ag-, TiO2- and CeO2-NP dispersions, used as models. NP characterization (size and morphology by TEM), hydrodynamic size and zeta potential were performed. Viability comparisons were determined in BEAS-2B cells. Cellular NP uptake and induced ROS production was assessed by TEM and flow cytometry, respectively. The obtained results show no important differences between frozen and fresh NP in their physico-chemical characteristics or their biological effects. This study is the first to demonstrate that there is no scientific evidence to dismiss the use of frozen NP, opening the door to the development of short- and long-term experiments with higher consistency, accuracy and reproducibility in a much shorter time and using a simplified procedure.*

The paper mentions dosimetry as important next step, and this was added in PATROLS T1.2
To enable parallel processing of ENM by invitro testing, the NANoREG SOP section 9.3 recommends flash freezing after dispersion, and thawing immediately prior to use. We decided to test and subsequently apply the flash freezing procedure in this deliverable. Prior to making this experimental design decision, we investigated whether the process of flash freezing would affect the dispersion quality of a variety of ENMs. Three materials were selected for in depth comparison of dispersion characteristics. The chosen materials- NM 212, NM 220, and Amorphous SiO2- were dispersed in triplicate as described in the methods section and then aliquoted. Most of the aliquots were immediately flash frozen. A non-frozen aliquot of each dispersion, termed “Fresh”, was measured immediately on DLS, diluted in media and incubated at 37°C for 1 hour, then measured using DLS and VCM. The flash frozen aliquots were kept for at least a week in a frozen condition (-78.5°C) before thawing in a hot sonicator bath, immediately followed by characterization. All procedures were done as described in the Methods section.

Table 1: *Z-Average and Effective Density values obtained for NM 212, NM 220 and Amorphous SiO_2 when the particles are measured either immediatelly after dispersion, or after flash freezing, one week storage, and subsequent thawing. N=3, *N=2.*

ENM	Z-Average (nm ± σ)				Effective Density (g/cm3 ± σ)	
	Primary Dispersion		1 hour Media Incubation			
	"Fresh" Dispersion	Flash Frozen Dispersion	"Fresh" Dispersion	Flash Frozen Dispersion	"Fresh" Dispersion	Flash Frozen Dispersion
NM 212	219.3±2.3	281.0±18.9*	261.2±3.8	242.6±2.6*	2.200±0.067*	2.214±0.029
NM 220	126.1±1.00	176.4±37.85	115.6±1.5	151.1±27.5	2.174±0.000	2.034±0.049
Amorphous SiO_2	148.3±0.4	230.8±26.8	1161.3±71.6	1169.1±155.2	1.156±0.004	1.156±0.013

We found that the Z-Average of the primary dispersion, as measured by DLS, was changed, usually with larger particles and a wider standard distribution (**Table 1**). The size distributions after 1-hour incubations in media were much less sensitive to the freeze-thaw process than the primary dispersions: the Z-Average is quite similar to the “fresh” dispersion. The differences were not pronounced in the effective density measurements, and the errors between triplicates were comparable (**Table 1**).

All of the above, together with the extensive validation by Vila et al, indicates that nanotoxicology experiments can use the freeze-thaw process.

However, there were also differences in size distribution that are not apparent from the Z-Average values. The primary dispersions tended to be larger and some ENM have populations of large aggregates, as opposed to others showing a simple shifting and widening of the size distribution. Even small contents of larger agglomerates have a profound effect on the delivered dose. E.g. the DG model, but also the direct integration of AUC sedimentation coefficients, integrate the entire size distribution, not only the Z-average. Hence, dosimetry needs to be determined on the exact sample prep that is also used for invitro investigations, and one cannot model dosimetry universally for either sample prep. Details will be report in D1.2.

References:

Vila, Laura, Laura Rubio, Balasubramanyam Annangi, Alba García-Rodríguez, Ricard Marcos, and Alba Hernández. 2017. 'Frozen dispersions of nanomaterials are a useful operational procedure in nanotoxicology', *Nanotoxicology*, 11: 31-40.