



## Live Webinar Q&A Sheet:

### Characterization of Lipid Nanoparticle Drug Formulations

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#### General FFF questions

*Q: What is the capacity of FFF? i.e. the volume that can be processed in a day.*

A: The sample capacity for a single run depends on the channel size; it ranges from a few micrograms (short analytical channel) to hundreds of micrograms or even milligrams of material (semi-preparative channel). Note that the relevant loading parameter is mass - the volume injected is variable, because the sample is concentrated during the focusing step, regardless of actual injected volume. In terms of total sample throughput per day, consider that one fractionation run takes between 30 minutes to one hour, and the system can operate unattended to perform run after run, 24/7.

*Q: On what type of membrane was the separation carried out? Will the separation change with a different membrane composition?*

A: Most frequently, membranes of regenerated cellulose or polyethersulfone are used. The membrane type can have an influence on resolution and recovery if the sample interacts strongly with the membrane surface. One example would be electrostatic repulsion or attraction in the case of a charged sample which is analyzed in a low ionic strength carrier solution. These conditions can be easily optimized to prevent unwanted interactions. In principle any sheet membrane can be used, simply cutting out a piece of the correct shape and dimensions using a template, but 95% of all applications are done with RC or PES membranes.

*Q: What is the pore size or molecular weight cut-off (MWCO) of the membrane used in the channel?*

A: These experiments generally used membranes with MWCO of 5 – 10 kDa. Membranes of different materials (e.g. regenerated cellulose, polyethersulfone) and MWCO (e.g. 5, 10 and 30 kDa) are commercially available.



*Q: How to avoid the adsorption of the sample to the FFF membrane?*

A: If the sample properties such as zeta potential and hydrophobicity are known, one can select the optimal membrane material and buffer (e.g. pH). The standard membranes have a slight negative surface charge, so a slight negative charge of the particles normally are advantageous. For some particles, it might be advantageous to add a very small amount of surfactant to the carrier liquid.

*Q: Can we predict the size of the particle of micrometer range?*

A: Once particles are in the micrometer range, they elute in the so-called steric mode. Here no theory is available to predict the retention time, but the channel can be calibrated with particle size standards and the size calculations is very similar to hydrodynamic chromatography (HDC).

*Q: Does concentrating the analyte near the membrane, at the bottom of the channel, decrease sample recovery since the outlet is on the channel top? How to achieve the maximal sample recovery?*

A: Positioning the channel outlet at the top does not lead to sample loss, because the laminar flow always transports the sample towards the outlet. Sample loss can only occur if the sample is adsorbed to the membrane surface or passes through the pores.

*Q: How does the data compare with the 250  $\mu\text{m}$  spacer?*

A: The change in fractionation using a 250  $\mu\text{m}$  spacer, compared to the standard 350  $\mu\text{m}$  spacer, can be calculated with Wyatt's SCOUT simulation software. Generally, the retention time will be halved with respect to the 350  $\mu\text{m}$  spacer under otherwise similar conditions.

*Q: Do you have any suggestions to optimize AF4 so that the run time can be reduced to be less time-consuming (45-70 mins per run)? Would such a long run-time cause sample LNP degradation?*

A: For homogeneous samples (e.g. BSA), the analysis time can be rather short (e.g. 30-45 min). Also, if one does not need complete separation, but only separation of two, well-defined and narrow-sized fractions, one can keep the analysis time short (e.g. DSPE-mPEG micelles can be separated from liposomes in a run of 30 min). If adequate size analysis of a sample with broad size distribution is needed, analysis time can become longer. In any case, one has to ensure physical and chemical stability of the sample over the run time. This is, however, normally not an issue (drug release is not seen as "instability" in this case).

*Q: Does one needs to make few "dummy injections" to saturate the membrane when starting the separation sequence on AF4?*

A: The membrane should be sufficiently rinsed with the carrier liquid before analysis. We rinse it normally overnight. One may also discard first analysis, if there is a difference to the subsequent



results. So far, we did not have problems with that with one exception. In this case, we used a new membrane.

Q: *In your case, what is the effect of the mobile phase on the recovery, separation, selectivity and size distribution? What is the best mobile phase?*

A: Concerning selection of the carrier liquid, the first prerequisite is physical stability of the particles. Also, a certain negative charge on the particles/molecules will be advantageous with respect to aggregation/destabilization and membrane interaction. Normally we keep salt concentration low and adjust pH. AF4 is rather flexible concerning pH range and salt concentration. For certain particles, addition of a small amount of surfactant may be advantageous.

Q: *How do the opposing flows focus the sample in bands?*

A: Focusing in a narrow band occurs because the flows come in from both sides of the channel and are balanced to meet beneath the injection port; in this position the path lines are perpendicularly directed downward. Therefore the sample is concentrated towards the membrane and focused into thin band, staying stationary with respect to the position in the channel.

Q: *What are the NP standards used? Material type, part number, vendor, etc.?*

A: The NP standards are polystyrene NP standards with certified particle sizes (Duke standards). The standards have been measured individually as well as in mixture.

*J. Kuntsche, K. Klaus, F. Steiniger. Size determinations of colloidal fat emulsions: A comparative study. J. Biomed. Nanotechnol. 5 (2009) 384-395.*

*J. Kuntsche, C. Decker, A. Fahr. Analysis of liposomes using asymmetrical flow field-flow fractionation: Separation conditions and drug/lipid recovery. J. Sep. Sci. 35 (2012) 1993-2001.*

Q: *What is the resolution and recovery of the system?*

*Is membrane passivation required?*

*Using chromatographic separation analysis, what typical resolution R is achieved for the lipid nanocarriers?*

A: Resolution is widely variable and can be estimated using FFF theory (implemented in Wyatt's SCOUT software). Recovery should be generally close to 100%, but it depends on the sample type and experimental conditions. If the sample tends to adhere to the membrane because it is positively charged or hydrophobic, it may be possible to passivate the membrane by injecting the sample several times, or by coating it with BSA.



Q: *Is the concentration determination in flow precise enough to determine the size by MALS since traditionally the measurements are concentration-dependent? Are there differences between in-flow measurements and in-cuvette measurements?*

A: Analysis of size by MALS is not concentration-dependent (though analysis of molar mass is) and is not flow-rate dependent. Though not needed for size determination, concentration measurements in standard online instruments (used to determine drug loading) are very precise under flow.

You may be thinking of size determination by dynamic light scattering, which does vary when concentration is high enough to reach the multiple-scattering regime; typically that does not happen after FFF separation, which significantly dilutes the sample. In any case, multiple scattering has little effect on MALS analysis unless the sample – after separation - is extremely turbid.

#### Questions regarding liposome analysis

Q: *Is there a recommended sample concentration and sample load size that is optimal for LNP on the AF4 channel?*

A: In general, the answer depends on the sample size and size distribution, and channel geometry. For most LNPs, only a few micrograms of sample are needed to have sufficient high signals in the light scattering detector (even if they are heterogeneous and spread out during fractionation); in fact, too much sample can saturate the detector (especially for homogeneous samples where all the material is in a single, narrow peak). However, depending on the carrier solvent and lipid composition as well as high polydispersity, the scattering efficiency may be lower and more sample, e.g. tens of  $\mu\text{g}$ , may be required.

Q: *How does dilution change liposome particle size? If we are separating, we wouldn't be measuring particle size at the same conditions as the desired conditions.*

A: As long as we avoid osmotic effects, there is no change in size (size distribution) upon dilution of lecithin liposomes.

*J. Kuntsche, C. Decker, A. Fahr. Analysis of liposomes using asymmetrical flow field-flow fractionation: Separation conditions and drug/lipid recovery. J. Sep. Sci. 35 (2012) 1993-2001.*

Q: *When calculating MW and PDI, what model do you use for LNPs: sphere, Lorenz-Mie, or coated sphere?*

A: The model used for MALS analysis depends on the sample. For liposomes the coated sphere model is used, while for emulsion droplets we used the sphere model. Lorenz-Mie is also appropriate as it assumes a spherical particles shape, though generally lipid nanoparticles are



small enough that Mie scattering models are not required. For non-spherical particles, we used the Berry or Debye models applying a higher fit order.

The PDI calculation is from DLS analysis (method of cumulants). Based on MALS size distributions one can also define polydispersity, but the usual MALS definition of  $M_w/M_n$  or  $R_w/R_n$  does not translate well to PDI from DLS; a more relevant calculation might be the relative standard deviation of the size distribution (if monomodal).

*Q: In the autoclaving study, the poloxamer MW was generally below 10 kDa. Did you recover all of the poloxamer, or is it possible some of it went through the AF4 membrane?*

*A:* There are membranes with different MWCO available. For the experiments with poloxamer-stabilized lipid nanoparticle dispersions, membranes with 5 kDa MWCO were used. To determine the recovery, poloxamer solutions with known concentration were injected directly (e.g. without a separation channel). The recovery after AF4 separation under applied conditions was > 90% in all experiments.

*K. Göke, E. Roese, A. Arnold, J. Kuntsche, H. Bunjes. Control over particle size distribution by autoclaving poloxamer-stabilized trimyristin nanodispersions. Mol. Pharmaceutics 13 (2016) 3187-3195.*

*Q: Is it possible to calculate the NP surface coverage by poloxamer according to the shape and size, for example a complete or partial monolayer?*

*A:* Generally, it will be possible to estimate the NP surface coverage by poloxamer, as the number of particles can be calculated from MALS data (assuming a similar particle structure, e.g. spherical for the whole sample) and the poloxamer amount is determined by the dRI detector. This has, however, not been done in the study.

*Q: What are the differences between the different types of lipid nanocarriers?*

*A:* The differences between the different types of lipid nanoparticles are both properties of the lipids (e.g. polarity) and structure of the nanoparticles (solid, liquid, liquid-crystalline) which will have an influence on drug incorporation and release (for a given API) and in-vivo fate (stability, release). One type of lipid nanoparticle is not necessarily the best for all API/purposes, but the most promising type depends on physico-chemical properties of the API, the intended administration route and release properties.

*Q: Which MALS model(s) has been used and why for all these lipid formulations?*

*A:* Which MALS model is used, depends on the sample. For liposomes (unilamellar), we apply the "coated sphere" model, for lipid emulsions the "compact sphere" model.



Generally, if one know the shape/structure of the particles, one should chose the corresponding MALS method (if available). Also Lorenz Mie assumes a spherical particle shape. For non-spherical particles, the fitting of MALS data becomes more challenging. We are apply in this cases the “Berry fit” or “Debye fit” and adjust the fit-factor.

In all cases, on should check adequate separation as all MALS fitting assume a homogeneous (e.g. monomodal) sample fraction. E.g. if two particles have the same diffusion properties but are different in their structure or composition (resulting in different scattering properties), they cannot be separated by AF4 and it may be difficult to fit the data.

*J. Kuntsche, K. Klaus, F. Steiniger. Size determinations of colloidal fat emulsions: A comparative study. J. Biomed. Nanotechnol. 5 (2009) 384-395.*

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#### Questions regarding drug quantitation

**Q:** How did you measure drug recovery after AF4 in Example 3?

**A:** Drug recovery was calculated by comparison of the injected mass of API and the API amount determined after AF4.

In the measurements of radio-labeled samples, the liposome fractions were collected after AF4. Radioactivity was measured off-line (scintillation counting) of the original liposomes, collected fractions, samples taken from the cross flow and from the membrane.

For the transfer studies, p-THPP amount (model API) was quantified on-line by absorbance measurements after correction for scattering effects. The p-THPP concentration was determined in original samples by (off-line, in solution) HPLC or absorbance measurements. Several controls were carried out (e.g. incubation mixture with non-loaded liposomes, loaded liposomes alone, etc.). In addition, for selected samples off-line quantification of p-THPP in collected samples fraction was also carried out.

*J. Kuntsche, C. Decker, A. Fahr. Analysis of liposomes using asymmetrical flow field-flow fractionation: Separation conditions and drug/lipid recovery. J. Sep. Sci. 35 (2012) 1993-2001.*

*A. Hinna, F. Steiniger, S. Hupfeld, M. Brandl, J. Kuntsche. Asymmetrical flow field-flow fractionation with on-line detection for drug transfer studies: A feasibility study. Anal. Bioanal. Chem. 406 (2014) 7827-7839.*



Q: *How do you subtract the particle's effect on UV? Just by subtracting the UV peak area of the empty liposome?*

A: Yes. We run non-loaded liposomes under same condition and carry out same data analysis, then subtract the AUC (absorbance detector area-under-the-curve) of non-loaded liposomes from the signal from loaded liposomes. This can be done directly in ASTRA or by exporting raw data and performing signal integration, after baseline subtraction, in math software.

Q: *Does the UV scattering correction for on-line quantification of drug loading assume the same number of empty and filled liposomes? If you don't know a priori that they are the same, how could you determine the quantities of each?*

A: For on-line API quantification, a control sample (e.g. liposomes) without API with similar composition, size and size distribution (thus similar number of particles) is needed. This will normally be an approximation. However, as long as the contribution of absorbance from the API is much higher than contribution of scattering effects to the overall signal, this is working well. It needs to be evaluated for each API (comparison of signal from non-loaded and loaded liposomes).

*A. Hinna, F. Steiniger, S. Hupfeld, M. Brandl, J. Kuntsche. Asymmetrical flow field-flow fractionation with on-line detection for drug transfer studies: A feasibility study. Anal. Bioanal. Chem. 406 (2014) 7827-7839.*

*A.H. Hinna, S. Hupfeld, J. Kuntsche, M. Brandl. The use of asymmetrical flow field-flow fractionation with on-line detection in the study of drug retention within liposomal nanocarriers and drug transfer kinetics. J. Pharm. Biomed. Anal. 124 (2016) 157-163.*

Q: *What are the assumptions and limitations of the scattering correction to on-line quantification of drug content in liposomes? If the size changes with drug loading, does that impact the result?*

A: The assumption is that the scattering effects of control sample (e.g. non-loaded liposomes) is the same as the API-containing sample. Several control samples are used, e.g. loaded and non-loaded liposomes without acceptor phase to check general recovery, mixture of non-loaded liposomes with acceptor phase to monitor changes in size during incubation). Changes in size (or size distribution) during incubation can be easily monitored by MALS analysis.

The limitations of this approach are the extinction coefficient (should be high) and wavelength of maximum absorbance (should be at high wavelength) of the API, scattering properties of the particles (should be low) as well as (for drug redistribution studies) drug release properties into the water phase. If the drug is released too rapidly into the water phase, it will be lost during AF4.



Q: *There seems to be a shift in elution time between empty and loaded liposomes. Is that a result of changing size or interaction with the AF4 membrane?*

A: The signal (slide 22, from absorbance detector!) is very small for the non-loaded liposomes. When plotting the data on different scales, elution time/interval is similar for the empty and loaded liposomes.

Q: *How does log P affect drug recovery?*

A: For lipid nanoparticles (drug release due to drug diffusion and partitioning), the recovery will be lower with decreasing logP as the drug (as long as it has a certain water solubility) will be rapidly partition into the water phase and be lost over the membrane (efficient “ultrafiltration” during focusing in AF4).

*J. Kuntsche, C. Decker, A. Fahr. Analysis of liposomes using asymmetrical flow field-flow fractionation: Separation conditions and drug/lipid recovery. J. Sep. Sci. 35 (2012) 1993-2001.*

Q: *Can AF4 be used to quantify drug release from formulations other than liposomes, for example from a polymeric nanocarrier containing a water-soluble drug substance?*

A: This should be possible as long as the drug is not released too quickly and the contribution of API absorbance is distinctly higher than the scattering effects coming from the polymeric particles. Generally, it is possible to connect other detectors (e.g. fluorescence) to the AF4 system.

Q: *What other possible mechanisms could explain the redistribution of the drugs/small molecules? You mentioned collision between the vesicles-have you been able to confirm this?*

A: Other mechanisms may, of course, include direct interactions between the donor and acceptor phases, such as fusion, lipid exchange, etc. - this can be expected e.g. upon mixing donor and acceptor liposomes with same lipid composition.

Assuming that there is no such destabilization, drug release/redistribution will generally be by diffusion through the water phase and by collision of donor and acceptor particles. For APIs that are insoluble in water and having a very high logP (such as p-THPP), diffusion via the water phase will not occur under normal experimental conditions - the volume of water phase needed to detect an API logP of 9 is very high. The assumption of a p-THPP transfer mechanism by collisions is supported by the results of dialysis experiments that we carried out, where the acceptor and donor phases were separated by a dialysis membrane (unpublished results). In this setup, no drug release or transfer was observed over a period of weeks, using either buffer or liposomes as the acceptor.



*Q: Does the analysis require calibration with the free drug prior to the drug quantification inside the liposomes?*

A: The drug's extinction coefficient at the selected UV/Vis wavelength should be known. If not known a priori, it should be measured – you might consider this “calibration with free drug”

*Q: Is the drug's UV/Vis absorbance inside the liposome the same as that of free drug?*

A: That depends on the specific absorbance properties of the drug, since the extinction coefficient may be different in different solvents. For on-line quantification, an on-line calibration curve with liposomes with known drug concentration is necessary, measured by direct injections of known volumes of loaded and non-loaded liposomes into the UV/Vis detector.

*Q: Is it possible to use these techniques for liposomes containing drugs that are also fluorophores and highly fluorescent?*

A: The p-THPP is a fluorescent compound (excitation wavelength 420 nm), but this did not cause any problems in size determination or on-line quantification. However, if compound fluorescence is excited at the MALS laser wavelength of 660 nm, fluorescent emission will combine with scattered light to skew the angular scattering measurements. In this case, fluorescent-blocking filters may be installed in front of the detectors. Another option is to change the MALS laser to one that operates at 785 nm which may prevent fluorescence. Sometimes both solutions are necessary.

If fluorescence is not excited by the MALS laser but only at shorter wavelengths, an online fluorescence detector can be used to quantify the drug.

*Q: You mentioned that monitoring of size during the drug release study is an advantage of AF4. Does that 'size' refer to the size of the liposomes or something else?*

A: As long as the sample has  $R_g > 10$  nm, the size can be determined by MALS for all sample fractions. In fractions containing molecules that are too small (e.g. albumin), the molar mass can be monitored instead of the size – using MALS plus the dRI detector for the concentration measurement.

*Q: Is there a way to calculate the RNA and lipids in the particle, similar to that of the protein-polymer conjugate?*

A: When a control sample (e.g. liposomes with same composition and size but without RNA) is available, this should be possible. Also, MS could be connected (or collected sample fractions can be submitted to LC/MS) to quantify lipid and payload, e.g. RNA.



*Q: How important is the monitoring of size during drug release study?*

A: A change in size indicates destabilization that may influence drug release properties. Size and size distribution should therefore be monitored.

*Q: For drug release analysis, if it is a small molecule and water soluble, would you have to take into account the free fraction (i.e., the fraction not absorbed to serum proteins)? How to recover the free drug by AF4?*

A: Yes, the quantity of drug released into the water phase (e.g. loss of drug during separation) must be taken into account for accurate data interpretation, especially if a large amount of drug is lost. Notably, this would also provide valuable information concerning in-vivo behavior. If there is no strong binding of the drug to serum proteins in-vitro (e.g. as determined by AF4 separation of samples incubated in serum), I would not expect to see strong binding to serum proteins in-vivo either, since the “aqueous phase” itself constitutes a large sink for the drug molecules.

Concerning experimental conditions in AF4, most drugs that are released into the water phase are lost during focusing (this can be considered a “forced” ultrafiltration). Such losses can be reduced by using the frit-inlet channel, where the sample is not subjected to a focusing step during injection. In our experiments, we saw higher recovery of drug (or other small compounds) which were lost to some degree during focusing in a conventional channel.