Live Webinar Q&A Sheet:

Optimizing microfluidic LNP formulation with in-line particle size and concentration measurements

The recorded webinar may be viewed from the RT-MALS webinar page. Additional information on SEC-MALS, DLS, CG-MALS, and FFF may be found in the Wyatt website Resources section under Webinars, Application Notes, and Bibliography, as well as on the corresponding Product page and Solutions page of our website.

The responses to the questions below were prepared jointly with the webinar presenter Dr. Jeremie Parot of SINTEF Industry, to whom Wyatt extends our sincere appreciation for his support. Please contact info@wyatt.com with any additional questions.

Calypso and nanoparticle production

Q: *What is the largest volume of a nanoparticle solution the Calypso can make?*

A: In principle, the Calypso® composition-gradient system can make any amount you like. The syringe pumps' valves enable automated loading from a reservoir of any size, so you can have the software keep reloading and dispensing.

The maximum volume of a single dispense depends on the syringe volume, flow-rate ratio and pump configuration. For example, if you install 5 mL syringes on all three pumps and configure the Calypso so that pumps 2 and 3 combine in reciprocating fashion (while one loads the other dispenses, then they switch), and the flow-rate ratio is 1:3, a single load and dispense cycle could deliver 20 mL (if the flow rate ratio is 1:4 then a single dispense would be 25 mL). If you need more than that, just program the software to repeat as many times as needed.

Q: *Can the Calypso be integrated with a fraction collector?*

A: At this time we do not offer such integration, but the Calypso does have the necessary connections to do so, and it would definitely be on the agenda to add that capability to the CALYPSO™ software.

Q: *You mentioned that it is possible to produce a sample volume of 0.1 mL with the Calypso. Is that enough to get a good inline light scattering measurement? Could you recover the sample at the end?*

A: A volume of 100 µL is somewhat borderline for the ultraDAWN® RT-MALS instrument, because its flow cell volume is 63 µL and the tubing leading up to it and then out to the collection vial will add another ~ 150 µL. It would be better to use the Calypso's inline dilution option to dilute 2-fold with stabilization buffer, for a 200 µL plug. Then, after mixing the solutions, either flow ~ 100 µL

of the dilution buffer (to ensure that the particles fill the flow cell) and stop the flow for optimal DLS measurements, or use the dilution buffer pump to flow slowly (so you collect at least a few data points), pushing the plug of LNPs through the flow cell while measuring MALS and DLS. Then push more buffer in order to fully recover the plug.

Q: Can the Calypso make various LNPs easily?

A: Like similar microfluidic systems, it is generally agnostic to the types of LNPs or other nanoparticles created through microfluidic mixing. The key is getting the right mix of lipids and RNA into the system.

Q: What's the minimum flow rate we can maintain to get stable LNP formulation?

A: This depends on the mixer. For the 0.5 mm i.d. tee-junction mixer, it's probably best to stay above 3 mL/min. The Calypso itself can maintain very low flow rates, the limit of which will depend on syringe size – with 1 mL syringes you could certainly maintain stable flows below 0.05 mL/min

Q: Which is preferable for optimal LNP production, inline dilution or dialysis? Can some form of flow dialysis like tangential flow filtration be coupled to the microfluidic system?

A: The Calypso can perform in-line dilution with a suitable stabilizing buffer that increases pH and reduces lipid concentration. In this configuration, two of the three syringe pumps serve for mixing and LNP production in the first mixer, and the third pump for dilution in the second mixer.

Inline dilution is a good first step to stabilize the nanoparticles, but it is insufficient for long-term stability (the organic solvent is not removed) and so a true dialysis should be done. It may be feasible to couple a small single-pass TFF cartridge to the Calypso, though this has not been tested as yet.

Q: If you develop a suitable LNP-RNA formulation with Calypso and ultraDAWN, how would you scale it for clinical production?

A: Scaling a microfluidic formulation to production generally means taking the selected mixer and flow conditions, implementing them on a continuous pumping system (as opposed to syringe pumps), and replicating this many times in parallel, combining the streams for a large total output flow. Generally you will have to tweak flow conditions a bit for optimal results.

The mixer identified with Calypso flow parameters can be transferred in this way to scale-up. An ultraDAWN can be incorporated on the final flow path to monitor size and concentration, either in-line for flows up to 150 or 200 mL/min, or on-line (pulling off a small fraction of the stream, which can be recovered) for larger flow rates. Wyatt is developing in-line instruments for higher flows as well.

Q: *How does the Calypso connect to the MALS/DLS instrument?*

A: It is simply a 1/16" PEEK capillary tube with a ¼"-28 flat-bottom fitting on the Calypso end and a 10-32 cone fitting on the ultraDAWN end.

Q: *Some lipids have higher transition temperatures and can thus crystallize upon mixing the organic-lipid-mixture with the aqueous buffer. To prevent the crystallization, can the syringes or mixers be heated within the system (e.g. to 60 °C)? Or is the mixer seated outside the Calypso system and heated separately?*

A: The Calypso does not have any internal heating capabilities. The mixer may be situated outside the system, where you can control its temperature with an appropriate device. However, the ultraDAWN also does not have internal heating capabilities, so if the lipids may crystallize downstream of the mixer, you might not want the ultraDAWN in-line.

ultraDAWN

Q: *Is the ultraDAWN GMP-compliant for monitoring clinical LNP production?*

A: In terms of the instrument itself, the ultraDAWN's flow path can be sterilized with 1 M NaOH. Its enclosure is not suitable for spraying down, so it must be contained within a suitable cabinet. We do expect to have a fully GMP-compatible instrument in the future.

In terms of the software, ultraDAWN can be operated in two ways.

- ASTRA® light-scattering analysis software collects data during the process, but analysis only takes place after the run is complete. ASTRA is offered with native 21 CFR part 11 compliance.

- OBSERVER™ real-time software is suitable for real-time monitoring and control with multi-angle light scattering. OBSERVER is not currently offered with native 21 CFR part 11 compliance, but when integrated with an OPC-UA client, OBSERVER's user interface is locked; all control and data acquisition takes place via the client, which can also take care of compliance aspects like audit trails and data security.

Q: *Can the ultraDAWN be used with LNP-RNA production above 150 mL/min?*

A: Yes, it can, in several ways:

- The ultraDAWN itself is not actually limited to 150 mL/min, it can tolerate higher flow rates for in-line operation. Rather, the maximum flow rate is typically dictated by the backpressure that the process equipment can accommodate. At 150 mL/min of water, the ultraDAWN produces 0.7 bar of backpressure. An ethanol-water mixture is more viscous than water so

the backpressure will be higher. If the process equipment can handle it then you can probably go to 300 mL/min or more in the ultraDAWN.

- The ultraDAWN can be configured for on-line operation, using a pump that draws a small slipstream from the main process flow. While this will increase the response time delay between the measurement and the process, it accommodates an arbitrarily high process flow rate.

- We are developing versions of in-line RT-MALS instrumentation that can go up to several L/min of flow.

Q: *Have you any experience with polydisperse samples? How does the system respond?*

A: MALS provides a root-z-average mean-square radius for polydisperse samples, while DLS provides a z-average harmonic radius. Consequentially, radius by MALS is biased towards the larger end of the distribution while radius by DLS is biased towards the smaller end of the distribution.

Q: *When MALS is combined with DLS, the DLS measurements provide size and polydispersity, and MALS provides the LNP concentration, right?*

A: Both MALS and DLS provide average sizes, though they are averaged differently (see previous response about why they differ). Then DLS further provides polydispersity while MALS provides LNP concentration. Note that only MALS is suitable for measurements during flow.

Q: *Can ultraDAWN or other instrumentation automatically monitor the nucleic acid encapsulation efficiency?*

A: It would be quite a feat to monitor encapsulation efficiency without a separation step. This is probably not feasible in continuous monitoring, but should be possible using automated periodic sampling from a process stream followed by separation, quantifying the free nucleic acid. Wyatt does not offer such a system but they are available from various HPLC vendors.

Q: *In the data showing the effect of total flow rate and flow-rate ratio, the LNP size increased, but particle concentration did not decrease. Why does the particle concentration not decrease, as the total lipid quantity remains same?*

A: That would be true if the efficiency of LNP formation remains the same. However, a non-optimal lipid-RNA ratio or flow rate might lead to decreased LNP formation efficiency.

In addition, conditions that lead to larger polydispersity and 'blebbing' could render the analysis (which relies on assuming relatively narrow distributions of true spheres) less accurate.

Nanoparticle characterization

Q: *Why does the size determined by MALS differ so much from the size determined by DLS?*

A: For spherical particles of a uniform size, the geometric radius determined by MALS is generally very close to the hydrodynamic radius determined by DLS. The discrepancies arise for polydisperse particles, where the averaging effects of MALS and DLS are quite different: MALS gives the square root of the z-averaged square radius, while DLS gives the harmonic z-average of the radius. In a polydisperse ensemble of spheres, MALS will give a larger size than DLS.

In addition, when the particles are not spherical, MALS and DLS generally give different results because they measure quite different properties. MALS measurements indicate the distribution of mass relative to the center of gravity of the particle, whereas DLS measurements indicate the diffusion coefficient.

Q: *On slide 42, why is the order of R_h as determined in-line opposite from the order of R_h as determined off-line?*

A: After the particles were produced and collected outside the Calypso-ultraDAWN system, they were subjected to dialysis involving a change in pH and buffer, and stabilizing excipients, as well as Oswald ripening. These processes may have a different impact on the particles depending on the formulation conditions.

Q: *The FFF analytical technique indicates that R_g is less than R_h, but the in-line results have the opposite (R_g larger than R_h). Do you know why?*

A: Normally for a polydisperse sample you would expect $R_g > R_h$, as was observed inline. If you review carefully the FFF-MALS graph you will see that there is a tail in the LS fractogram of larger particles that was not included in the region selected for analysis. While this tail contains a very small fraction of the total particle mass, it has a large effect on the z-average R_g, and therefore was excluded from the analysis. The final R_g value from FFF is more representative of the particles of interest, but obviously this selection is not available for in-line monitoring.

Q: *We use DLS to validate our LNP. During this we must identify an ideal concentration for accurate measurement of size. Is MALS more tolerant of sample concentration?*

A: Yes, MALS is more tolerant than DLS of sample concentration for size measurements. MALS has a very large dynamic range and is not subject to multiple scattering effects or (with attenuators installed in an ultraDAWN) to detector saturation. However, you should also recognize that dilution is important for eliminating actual particle-particle interactions that impact measured size and polydispersity. Plus, MALS cannot quantify polydispersity in a batch measurement.

DLS with roughly 1000-fold dilution is probably your best bet for doing so in batch. It would be quite straightforward to set up a dilution series, with replicates, in a microwell plate and use a DynaPro® Plate Reader to test the effect of dilution so as to ensure optimal DLS measurements of size, polydispersity and particle concentration. For truly accurate and quantitative analyses of nanoparticle size distributions, the best option is FFF-MALS using an Eclipse™ FFF system with a DAWN® MALS detector.

Q: *Will TFF/dialysis need to be a separate offline operation? Also, in that case would the MALS-DLS instrument measure particle properties prior to ultrafiltration?*

A: For small-scale production with the Calypso, it could be feasible to add a single-pass TFF cartridge or perhaps a desalting column. This has yet to be tested. For larger scale production and the more typical multi-cycle TFF operations it is certainly feasible to implement RT-MALS at various points in the process in order to monitor concentration and potential size changes - this has been demonstrated with viral vectors and LNPs.

Q: *These inline measurements seem great to determine quite a few critical attributes. However, although LNPs in different samples could show a similar size distribution, how would you determine structure? How would you check if your LNPs are not 'blebbing' (with RNA going into an aqueous phase within LNPs) under different conditions?*

A: It is unlikely that inline measurements will be able to determine if LNPs are 'blebbing'. Offline techniques such as FFF are more suited to determining the detailed particle size and size distribution using MALS and DLS, and combining information from these two detectors should provide information about the particle shape, potentially highlighting the presence of 'blebbing' LNPS. The next step would be to apply high-resolution techniques oriented toward determining the structure of the particles such as cryo-EM.

Q: *What is the best method for measuring RNA concentration?*

A: Various techniques can determine the RNA concentration from LNPs such as fluorescence kits, HPLC-UV or mass spectrometry. Depending on the type of LNP and RNA, and on your requirements in terms of accuracy and sensitivity, a difficult choice will have to be made to pick the appropriate technique and method.

ASTRA's *Nanoconjugate Analysis* method enables quantification of the encapsulated API, without removing the lipids, directly in-line with FFF or SEC separation, by combining UV, RI and MALS detection. Beyond just quantifying the RNA concentration, this method determines the encapsulated payload as a function of particle size, which is an essential quality attribute for evaluating biological efficacy.

Q: *How to determine the titer and convert it to particle concentration, i.e. the particle number per mL?*

A: Particle concentration (particles per mL) is determined by light scattering alone. Per the equation shown on slide 3 of the presentation, all you need is R(0) (the reduced Rayleigh ratio, extrapolated to angle zero), the refractive indices of the particle and the solvent, and the volume of the particle. The volume can be determined with MALS using a shape model like uniform sphere, or by DLS. Wyatt offers particle concentration analysis with its DAWN, miniDAWN, microDAWN and ultraDAWN MALS detectors as well as DynaPro Plate Reader and DynaPro NanoStar® batch DLS detectors. An independent titer analysis is not needed.

Q: *Which form factor model do you suggest for the angle dependency of lipid nanoparticles containing RNA?*

A: Well-formed LNP-RNA are essentially uniform spheres, and MALS data can be analyzed with the Rayleigh or Lorenz-Mie sphere models. These models are best when MALS is coupled to a size-based separation system or when the sample is fairly homogeneous in size. Poorly formed LNP-RNA with 'blebs' are not so spherical so a general R_g model, like first- or second-order Berry. may be more appropriate. When the particles are polydisperse and not separated by size (as is the usually the case during process monitoring with ultraDAWN), sphere models do not work well and an R_g model is recommended for z-average size determination.

Q: *How did you determine the encapsulation efficiency?*

A: Encapsulation efficiency is determined via a separation method like field-flow fractionation or size-exclusion chromatography. The unencapsulated API is separated from the nanoparticles and quantified, then either compared to the total quantity expected from the injected volume and formulation recipe, or to the amount of encapsulated API as determined by quantifying that amount, using in-line or on-line methods. ASTRA's *Nanoconjugate Analysis* method quantifies the encapsulated API directly in-line with FFF or SEC separation, by combining UV, RI and MALS detection.

Q: *How was AF4 recovery determined?*

A: Recovery in AF4 is determined in three steps: 1) inject a volume of the sample into the AF4 channel with no cross flow applied, and determine the area under the concentration signal curve; 2) inject the same volume of sample, this time applying the AF4 cross-flow profile, and determine the area under the concentration signal curve; 3) divide the results to obtain recovery.

General

Q: *If we already have one of the standard microfluidic systems for LNP-RNA formulation, would you recommend adding Calypso or ultraDAWN to our lab?*

A: Standard microfluidic systems are fine for getting up and running and quickly producing good LNP-RNAs, but in the long run they tend to be expensive and less productive due to the costs of consumables and the labor associated with manually loading each test run. You may need multiple models to address different productions scales. In addition, they do not offer integrated analytics. If you plan on developing more than one LNP-RNA formulation (or formulate other nanomedicines using this technology), and would like the flexibility to test and optimize your process with any commercial or proprietary mixer and produce any volume, then adding Calypso + ultraDAWN to your arsenal will be highly beneficial.

Q: *Does the diameter affect the potency of that drug substance?*

A: LNP size is critical to biodistribution and efficacy of transduction.

Q: *Have you done head-to-head comparison between Calypso and NanoAssembler in terms of their performance in making LNPs?*

A: A head-to-head comparison between Calypso and NanoAssemblr has been conducted using two different LNPS and no major performance difference were discovered.

Q: *Were the experiments performed with a single size of RNA strand (number of nucleotides) or different sizes? If different sizes were used, what was the effect?*

A: These experiments were mainly done with CleanCap® FLuc mRNA (5moU) from Trilink, composed of 1929 nucleotides. While the microfluidic method should work with various sizes of RNA, for each size and type you will need to optimize the lipid ratio, N/P ratio and formulation parameters like total flow rate and flow rate ratio.

Q: *Do you have any problems with RNAses in your systems? And how do you minimize these when the system is shared for analysis of other particles?*

A: By following good practices in the lab and by using a proper cleaning procedure between sets of experiments, we never experienced problem with RNAses in our system.

Q: *How and when to remove impurities during the microfluidic process?*

A: Microfluidic processes such as LNP production usually begin with pure solutions, e.g. purified lipids and RNA (and ethanol and different buffers), and therefore do not include much in the way of impurities. The use of TFF (or dialysis) after the formulation process should remove all the unused lipids, the only potential 'impurities' could be some free RNA molecules.