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Webinar Q&A Sheet

VLP Characterization: the Light Scattering Biophysical Toolbox

The recorded webinar may be viewed here. These questions were submitted by live viewers. Additional information on MALS, DLS, and FFF may be found on the Wyatt Library under Webinars, Application Notes, Featured Publications and Bibliography, as well as on the corresponding Product and Theory pages of our website.

Please contact info@wyatt.com with any additional questions.

VLP-specific

1. *What is the best method to assess enveloped VLP aggregation?*

For full VLP aggregate characterization, whether enveloped or not, FFF-MALS addresses the widest size range on a single platform and eliminates column interactions/filtering which could potentially remove larger aggregates from the sample. The primary drawback of FFF-MALS is dilution, which can cause the dissociation of weakly-bound aggregates.

For screening aggregation against different buffer conditions, or to maintain concentration, batch DLS is ideal. In particular, the DynaPro Plate Reader II can readily run through hundreds or thousands of conditions in a day, including temperature ramps for stability.

2. *Which of these methods can most accurately provide shape information in addition to size, and why? Can you provide a specific example of how one might determine if a VLP has oligomerized into an elongate structure?*

FFF-MALS-DLS provides the best benefit here, as it combines separation with multiple analyses. Separating on FFF, we can measure the molar mass and radius of gyration R_g of the monomer via MALS. The oligomers are further separated on FFF by hydrodynamic radius R_h, but measured by MALS. For a given subset of molecular weights (VLP trimers, for example) the R_g will be distinct for a linear vs. triangular oligomer, etc. and as described in the presentation, if we assume the particles are spheres

and know the R_g of the monomer, we can calculate the R_g of each configuration. At any elution time, we determine molar mass of the oligomer, and deduce the conformation from the value of R_g relative to that of the monomer.

This analysis works for small oligomers, but as the order increases there will be increasing overlap in the separation between different oligomeric orders that have configurations with the same R_h. For example, a globular cluster of many particles could have the same R_h as a linear arrangement of fewer particles. The two would elute at the same time and the analysis would break down.

Additional constraints on the shape can be acquired by adding an online DLS module and measuring R_h, simultaneously with molar mass and R_g.

3. Can samples containing both VLP and small molecules be analyzed? What technique/methods would be best suited to pick up signal from any remaining unassociated protein from the intact VLP signal?

This is definitely possible, as shown in the webinar. The most information would come from online analysis, either SEC- or FFF-MALS which quantify the amount of material present as dissociated subunit(s) and its oligomeric state. Addition of dynamic light scattering to this online measurement could yield insight into the disposition of the non-associated material, e.g. is it folded or denatured.

Batch dynamic light scattering can also provide limited information about multimodal samples (e.g. intact VLPs in the presence of "other" molecules). For example, as long as radii of gyration are > 3-5x different one can determine size distributions of each sub-compartment. However, batch DLS would not be suitable for quantifying or assessing oligomeric state of the non-associated species, or for reliable determination of relative fractions of each size.

4. What are the drawbacks of SEC-MALS in characterizing VLP compared to FFF?

The primary drawback of SEC is the relatively narrow resolution range afforded by a single column configuration. It is often necessary to use multiple columns to adequately separate VLPs and their oligomeric counterparts from higher-order aggregates. The situation is complicated further if non-associated subunits must be resolved/characterized. Because separation in FFF is governed by flow parameters, which can be easily changed for the analytical requirements at hand, it's possible to separate a wider size range without modifying hardware components. Furthermore, as separation happens in an open channel in FFF, large aggregates are not filtered from the sample and there is significantly less potential for VLP-substrate interactions.

5. Can you provide a solid distinction between mass spectrometry (MS) and MALS for determination of the mass of VLPs?

The distinctions differ somewhat with respect to the type of mass spectrometry employed (MALDI vs. ESI-MSMS, etc.). Traditional MS employing electrospray ionization is generally limited to molecules of several thousand Daltons, and may disrupt the quaternary structure. Newer MS methods have been developed to measure much larger molecules, but relatively fragile VLPs with masses in the tens of megaDaltons are still quite difficult to work with. MALS does not afford the exquisite precision of MS, but allows VLP properties (mass, size, association properties, etc.) to be measured in solution under native or altered conditions.

6. How could one tease out impurities in VLPs, say DNA/RNA versus protein content?

Exogenous impurities are separated from the VLP peak by SEC or FFF. If the DNA/RNA is transported within the VLP envelope, we employ 'conjugate analysis' to determine the relative abundance and molar masses of each subcomponent. This requires knowledge of their UV extinction coefficients and dn/dc values.

7. For batch mode DLS or MALS analysis of VLPs, what sample prep should be done to ensure removal of dust particles?

Dust which is much larger than the VLPs is removed by filtration or centrifugation. For example, VLPs with a radius of 50 nm should be filtered with 0.2 μm pore filter membranes. Small dust particles, comparable in size to the VLP, should have minimal impact on the results.

8. Does the sample medium effect analysis, e.g. Cell culture buffer?

For online applications (SEC/FFF-MALS), buffer/media components are only problematic if they co-elute with the VLP or otherwise impact fractionation.

In batch (unfractionated) experiments, all components in solution will contribute to the scattering signal, so lysates and other large impurities that have not been filtered out could impact the scattering signal; small molecules such as salts and sugars will have little effect. Furthermore, any component which significantly changes the refractive index of the medium may skew the analysis.

From a practical standpoint, in batch static light scattering, small molecules, amino acids, etc., can sometimes be factored out by using the same medium (absent VLP) as a blank. This is not possible for dynamic light scattering, where the autocorrelation function will be representative of all the species in the scattering volume. If the

components are different enough in size (~3-5x) from the VLP one can still use regularization fitting to monitor size distributions, etc.

9. How would dn/dc value be calculated for viruses to support light scattering analysis?

dn/dc is not needed if only size (R_g or R_h) is to be measured. For molar mass determination it is usually sufficient to use dn/dc of a typical protein (0.185 mL/g). If the VLP is itself a composite (i.e. contains nucleic acid, polymer assemblies, etc.) its dn/dc may be calculated as the mass ratio of the protein and modifier dn/dcs, or determined empirically. For particle number measurements the refractive index of proteins (a well-known quantity) can usually be used.

10. Can we determine the molecular weight of a virus like particle or the molecular weight of hybrid colloids (i.e. gold nanoparticle with a protein corona?) How?

In order to determine molecular weight by MALS, macromolecules or particles need to fulfill certain conditions on refractive index and size. In particular, the particles should be small compared to the wavelength of light, transparent, and have a refractive index not too different from that of the solvent. VLPs generally fulfill these conditions and so MALS readily determines their molar mass, even if they contain a payload such as DNA. Gold particles do not (obviously they are not transparent). For hybrid colloids the best you could probably do is assess the size difference between decorated and undecorated particles, and perhaps even utilize the relationship between rms and hydrodynamic radii to understand the corona.

11. Can these techniques be used to determine the degree of correct folding in a sample of VLPs?

No. these techniques do not provide any high-resolution information about secondary or tertiary structure, unless misfolding results in significant changes in size/geometry or molar mass (e.g. for partially intact VLPs). In the latter case, either DLS or SLS could provide some qualitative or semi-quantitative information.

12. What is the sensitivity limit for counting viral particles (lower limit of VP/mL)?

Typical sensitivity limits for VLPs would be 10^7 particles/mL.

13. Is there a significant difference in the characterization of an intact virus from that of a VLP using FFF-MALS?

Possibly. If the intact virus is enveloped it may interact with the FFF membrane, likewise if the VLP has been externally modified (with targeting moieties, PEG, etc., for example). Additionally, intact viruses are composites of protein and nucleic acid so their

dn/dc and extinction coefficients will be distinct from those of a VLP consisting solely of protein. These deviations must be accounted for when determining molar mass but will not impact size measurements.

14. Please expound on the results of the VLP dissociation (150 mM NaCl and 90 mM bicarb) results.

DLS was used to show the kinetics of dissociation while FFF-MALS was used to analyze the final products in different buffers. Please see A. Citkowicz et al., Analytical Biochemistry **376** (2008) pp. 163–172.

15. Regarding conformation from AF4: how hollow is "hollow" (i.e. you need to know the thickness of the shell). VLPs have a hairy-like appearance. Can other form factors be used to determine conformation (e.g. core-shell)?

MALS and DLS together can provide information about distribution of mass (R_g) compared to the physical dimension of the VLP (R_h). Assignments of "filled vs hollow" are qualitative and based on models. Alternative methods would be necessary for more robust characterizations.

Measurement Characteristics and Comparisons

16. What is the range for particles size? What are the R_g limits for the mini-DAWN TREOS?

MALS: The range of the DAWN for root-mean-square particle size (R_g) is ~10 nm to 500 nm, and even up to 1000 nm assuming shape-specific models and sufficient angular coverage. The miniDAWN 3-angle detector only allows for sizes between ~10 nm to 50 nm, and up to 150 nm assuming shape-specific models.

FFF Separation: 1 nm to ~1000 nm radius, and up to several μm with some tricks

Online DLS: the hydrodynamic radius R_h can be determined accurately from 0.5 nm to ~300 nm. The upper limit actually depends on the flow rate and detection angle; assuming a flow rate of 0.5 mL/min and maximum angle available on the DAWN HELEOS II, ~200 nm.

Batch DLS: R_h can be determined accurately from 0.2 to 1000-5000 nm, depending on the instrument.

17. What is the throughput of the instrument and the minimum amount (and volume) which can be analyzed?

As a general rule of thumb, the FFF-MALS runs are on the same time frame and require the same material as a comparable chromatography run, 20-30 minutes and 10-100 μg of sample. More complex separations may require additional time.

Since the scattering sensitivity is proportional to the M_w times the concentration, with larger M_w material (such as VLPs) far less material may be utilized for good r.m.s (R_g) results, even if the molar mass cannot be calculated because the concentration is too low to be measured reliably.

18. Is there a concentration range that is best suited for light scattering measurements with PALS, MALS or DLS? What happens if the concentration is exceeded? How do you determine what the concentration should be? What are minimal concentration limits for proteins in SEC-MALS?

MALS: Wyatt MALS instruments provide a very large dynamic range. Since MALS is usually used online, the maximum concentration range is usually determined by the chromatography rather than the MALS detector; while in batch mode we routinely measure antibody solutions in the range of ~100 mg/mL, chromatographic separation will only support ~ 1 mg/mL. On the low end, MALS can measure below 1 μg/mL protein: as low as 0.2 μg/mL of BSA, which is typical of a 0.1 μg injection in SEC. However, it is important to remember that the sensitivity of MALS is a function of the product of M_w and concentration of the sample, so for small M_w samples, you would need to inject proportionally more sample and vice versa for large M_w samples. On the rare occasion that the scattering intensity exceeds the saturation range of the detectors, the saturated signal will be obvious and the laser power may be turned down.

DLS: The dynamic range of DLS is much smaller than that of MALS, and it is less obvious when the signal exceeds its allowable range, so our DLS instruments employ both an automatic attenuation feature and laser power adjustments to make sure that the measurements are optimized. Usually this optimization process works well, but if the instrument cannot adjust itself because the degree of scattering is too high, a warning is noted in the experimental results. For proteins and other macromolecules typical ranges are 0.1 – 10 mg/mL; higher concentrations result in non-ideal scattering behavior which is not well understood even though it is easy to make the measurement. For particles this range is highly dependent on particle size and composition. High concentrations of large particles may result in multiple scattering, yielding incorrect results.

PALS: For proteins and similar macromolecules the measurement range is typically 1-100 mg/mL. For particles this range is highly dependent on particle size and composition, but the range of measureable concentrations is fairly large.

In general, whenever a doubt arises regarding the validity of results at high concentrations, it is a good idea to measure as a function of concentration. If no concentration dependence is found, then the result may be considered reliable; if there is a concentration dependence, the origin of this behavior, e.g. interactions (macromolecules) or multiple scattering (large particles) should be considered.

19. Can FFF and SEC MALS be run simultaneously so both soluble and insoluble aggregate can be characterized at the same time?

Simultaneous analysis using SEC-MALS and FFF-MALS is really not necessary as FFF-MALS can separate both soluble and insoluble species, as well as soluble from insoluble. A complete separation in one run is accomplished using a cross-flow gradient: as the cross-flow changes, first the lower size range is resolved, then the larger. Of course FFF-MALS eliminates the stationary phase which can interfere with the analysis of the aggregated material.

20. Do you have calibrants?

Calibration of MALS or DLS against size standards is not necessary. The flow cell in the MALS detector is calibrated with a universal liquid, toluene, in order to characterize the Raleigh ratio (the ratio of the scattered and incident light intensities).

21. What is the M_w range that can be measured by MALS and what specific sample information needs to be known? What is the M_w resolution?

The M_w range of the DAWN HELEOS II MALS instrument is from ~300 Da to ~10^9 Da. One item of specific information that needs to be known before analysis is the differential refractive increment, dn/dc, in the solvent used in the experiment. For proteins in most aqueous buffers this value is essentially constant to within 1-2%.

It is also necessary to know the concentration. In online measurements, if a refractive index concentration detector is employed (dRI) then this is sufficient; if a UV detector is employed, then you would also need to know the absorptivity of the sample at the wavelength used.

The resolution is ~ 1-2% and the absolute accuracy ~5%. Online, the resolution is dictated by the separation mechanism rather than the detectors.

22. Can these techniques be used to measure asymmetric particles (e.g. phage…rods of 6 nm by 1 µm long)?

There are several ways to characterize such particles. If they are known to be rod-like, they may be analyzed by MALS, and an analysis model included in the ASTRA software reports the length. If the shape is not known, simultaneous DLS and MALS measurements can help discriminate between possible shapes.

23. How does the particle count measured by MALS compare with other techniques such as MFI?

MFI and MALS actually do not overlap in size range, and so cannot be compared. Analysis of adenovirus counts by MALS has been compared to estimates derived from cryoTEM, AFM and other techniques, and they agree to within a few %. See Z. Wei et al, Journal of Virological Methods **144** (2007) pp. 122–132.

24. What are the relative advantages of SEC/FFF-MALS vs. dynamic light scattering (DLS) and NanoTracking Analysis (NTA)? Is continuous-flow DLS available for sub-micron particles and small nanoparticles?

FFF-MALS offers a host of advantages over batch techniques such as DLS and NTA, since it performs a true size-based separation prior to characterization by light scattering and other detection methods:

- Issues often encountered in batch techniques, e.g. the scattering from larger particles 'blinding' the system to smaller particles, do not exist in FFF-MALS.
- There is no need to optimize concentrations as in NTA.
- Several online detectors can be included in one analysis, e.g. UV, fluorescence, refractive index, MALS and DLS, and even ICP-MS, in order to achieve maximum characterization of nanoparticles.
- Information regarding conformation may be derived from the ratio of size to mass or the ratio of R_g/R_h.
- Information regarding composition may be obtained from the combination of UV and RI, fluorescence or ICP-MS.

Continuous-flow (online) DLS is available for the size range 0.5 – 300 nm, either by installing a WyattQELS DLS module in an online MALS detector, or by connecting the detection fiber from a DynaPro NanoStar cuvette-based DLS instrument to the MALS detectors.

25. How do MALS and DLS compare to qNano?

The qNano instrument is a batch technique that measures particles based on how they pass through an aperture, and so estimates an envelope size similar to R_h. The diameter range of the qNano is dictated by this aperture size and is from ~50 nm to 10 μM (www.izon.com/products/qnano/qnano/). Its limitations include small ensembles, interactions with the membranes, and the requirement to calibrate against known standards. A set of membranes may be needed to cover this range, and if the pore size is small large particles may cause clogging.

Light scattering measures particle size based on two first principle techniques: MALS measures the angular scattering profile to determine R_g, and only requires knowledge of the refractive index of the solvent; DLS measures the translational diffusion coefficient (D_t) and requires only the solvent temperature and viscosity to determine R_h. Both MALS and DLS can measure much smaller sizes than qNano but MALS does not reach the upper range of 10 μM.

Both MALS and DLS can be employed as batch techniques or downstream of any separation technique such as FFF or chromatography. While separation adds complexity, it also adds the opportunity to apply multiple characterization methods and measure large ensembles in one go.

26. Is dRI detection necessary for absolute size (M_w/rms radius) determination in SEC/FFF-MALS, or is UV sufficient?

Concentration is not necessary when determining the RMS radius with MALS.

In many cases, either concentration technique can be used to good result for the M_w determination. However, for samples such a proteins that have any post-translational modification or complex mixtures such as VLPs containing a protein component, you must employ both UV and dRI to adequately characterize the complex. This type of dual concentration characterization is referred to as "conjugate analysis" and is incorporated in the ASTRA software.

27. Is it possible to analyze a sample by FFF which should exhibit both elution modes (steric and normal) and, if so, what recommendations do you have?

In normal FFF, smaller particles elute earlier. Steric elution mode occurs with particles above 0.8 - 1 μm, and reverses the order of elution. Combined steric and normal elution happens with samples containing mixtures of large and small material, and could easily be misinterpreted without a first principle characterization tool such as MALS

downstream of the separation. The simplest approach would be a preliminary separation step of 'large' from 'small' by filtration or centrifugation.

CG-MALS

28. With respect to the functionality test (CG-MALS?) - is that really a TRUE functionality test? How does CG-MALS compare with SPR for affinity/interaction studies?

While CG-MALS is of course performed in vitro in purified solution (and not in vivo or ex vivo), it can be considered an excellent measure of functionality with regard to affinity and stoichiometry of specific antibodies binding to the VLPs. It is not a pulldown assay to determine if any antibodies bind, in the sense that SPR or other techniques might be used to screen for functionality.

Because CG-MALS requires no immobilization and can determine the true solution binding properties of the interaction it is preferable to SPR for fully characterizing the interaction of VLPs with antibodies in solution, or other types of macromolecular interactions. Absolute determination of molar mass by MALS as a function of composition means that different types of complexes are readily distinguishable, including cooperative and multi-valent effects. However, it is not suitable for small-molecule/protein binding due to the small change in molar mass upon complexation.

29. Can this technique be used to monitor protein-ligand binding? How do you use CG-MALS to examine concentration-dependent association of a single protein species?

Yes, CG-MALS has been used extensively for protein ligand binding. It requires the molar mass to changes by ~10% or more upon complexation. Please see

www.wyatt.com/CG-MALS and

www.wyatt.com/library/references/featured-publications/view/featured-publication-22.

In a CG-MALS analysis of self-association the Calypso is programmed to deliver a series of concentrations to the detectors, achieved by mixing a stock solution of protein with diluent. Each aliquot is injected into the detectors and the flow stopped to allow for equilibration. In equilibrium, MALS determines the weight-average molar mass of solution based on light scattering and concentration. When a protein self-associates, as the concentration increases, complexes form and the weight-average molar mass of the solution increases. Because CG-MALS measures molar masses directly, the form and value of the molar mass/concentration dependence is quite specific to the order of association (e.g. dimerization or tetramerization).

Webinars on CG-MALS are available at www.wyatt.com/Webinars/CG-MALS.

30. What kinetic timescales can the Calypso measure?

From seconds to hours.

General Application Questions

31. Can MALS be used to detect/quantify surfactant/micelles (e.g. Tween)?

Yes. In fact, MALS detection, along with dRI and UV detection is the most complete way to characterize and quantify this protein-surfactant complex. Our software utilizes the conjugate analysis method (MALS-UV-RI) to get a M_w distribution of the complex, the core protein and the modifier.

32. For conjugate analysis – is it feasible to calculate the amount of protein and nucleic acid using the protein conjugate algorithm? What is a suggested dn/dc for the nucleic acid?

This is technically feasible but sometimes difficult because dn/dcs for protein and nucleic acid are similar. Depending on solvent and wavelength of light the dn/dc for DNA and RNA can range from ~0.170-0.180 mL/g and ~0.160-~0.170 mL/g, respectively.

33. Can intrinsic viscosity measurements help characterize structural changes in the molecule?

Probably not. In extreme cases a change in intrinsic viscosity might indicate a very significant change in axial ratio or loss of symmetry, but there are better techniques for probing structural perturbations.

34. What are the impacts of pressure and flow parameters on the analyses (i.e. how would this impact non-rigid structures)?

The impacts of pressure are negligible. Some large non-rigid structures may exhibit abnormal elution properties in FFF, however, this will not impact molar mass results.

35. Can DLS be combined with SEC to determine M_w, chain length and branching of starch? If not, what would you suggest?

Absolutely. Please see our recent application note:

Branching Revealed: Characterizing Molecular Structure in Synthetic and Natural Polymers by Multi-Angle Light Scattering and the associated webinar.

36. What causes different M_w results when using UV vs dRI? Why is the result different across the peak (i.e. not horizontal)?

For a homogenous sample (i.e. no co-elution) the results should be the same with either concentration source assuming the dn/dc and extinction coefficient are accurate. If the peak represents a mixture of species (e.g. modified and unmodified VLPs) each subcomponent may have distinct dn/dcs (and/or extinction coefficients) and mass results might deviate if there is a slight degree of separation between the species. In general, assuming that alignment and band broadening parameters are accurate, non-horizontal molar mass profiles indicate sample heterogeneity or polydispersity. This can result from contaminants, partially dissociated VLPs, fragmentation, etc.

37. Recommendations to separate particles in plasma, i.e low density lipoproteins vs circulating exosomes.

Please see R. N. Qureshi, W. Th. Kok, P. J. Schoenmakers, "Fractionation of human serum lipoproteins and simultaneous enzymatic determination of cholesterol and triglycerides", Analytica Chimica Acta, 654(1), pp. 85–91 (2009) DOI: [10.1016/j.aca.2009.06.060].

38. Do you have a literature reference for the liposome example presented? Please see our application note:

www.wyatt.com/files/literature/app-notes/fff-mals/liposome-fff-qels.pdf

39. For SEC-MALS do you use a solvent similar to cell cytoplasm?

SEC-MALS is performed in standard chromatographic mobile phases such as PBS or bis-tris buffers. These may be matched to the cytoplasm in terms of pH and ionic strength.

DLS and MALS Questions

40. Do DLS and SLS give the same size results?

DLS is used to derive the hydrodynamic radius, R_h, which is closely related to the 'envelope' of the particle, while MALS measures the root-mean-square radius R_g which

depends on how the mass is distributed throughout the particle's volume. Whether or not they provide the same value depends on the conformation of the sample including the shape and mass distribution. If the particle is a spherical shell, all the mass is concentrated in the envelope and the sizes are identical; if it is a uniform sphere the 'average' mass is in the interior of the sphere and so $R_g < R_h$. The ratio of R_g:R_h may be calculated theoretically for various cases such as rods or dendrimers, and can help determine the structure of the particle.

41. Is multi-angle static LS (MALS) more sensitive than fixed-angle (90°) static light scattering for a given analyte? Why or why not?

It is not so much a matter of sensitivity, but the size of the analyte. Macromolecules and particles with a radius greater than about 10 nm, including VLPs, exhibit anisotropic scattering (intensity varies with angle) while smaller species such as monomeric proteins exhibit isotropic scattering (intensity is independent of angle). The angular dependence encodes information on the size (R_g). The molar mass information is contained in the scattering at zero angle, but it is impossible to put a detector at zero (it would be blinded by the laser beam). If the scattering is isotropic then any angle – e.g. 90° - will do, while if the scattering is anisotropic the solution is to measure at multiple angles and extrapolate to zero.

With that said, there is always a sensitivity gain in making measurements with more detectors, even if the scattering is isotropic.

42. Do we need to account for sample viscosity in the calculations?

Solvent viscosity must be known for DLS analysis, but not for MALS. Typical values for a vast suite of solvents, along with thermal models for temperature studies, are embedded within DYNAMICS software. Custom solvents can also be added, for example if a formulation buffer has a significant excipient content then the solvent viscosity must be entered manually.

43. For R_g/R_h comparisons, does the MALS instrument also collect DLS data or must separate measurements be performed?

All Wyatt MALS instruments may be equipped with an onboard 'WyattQELS' DLS module, for simultaneous MALS and DLS measurements in the same flow cell.

44. For DLS, how much optimization for acquisition time is usually required for characterizing a new analyte?

Generally speaking for new or unknown samples, we employ a scan time of 10 seconds and then make 10 measurements of the sample. From these results we can quickly determine if the scan time or number of scans can be altered to better characterize the sample or to streamline the analysis. In either case, there is an automation calculator built into the software which will give you a good starting estimate of the scan parameters based on your sample size and concentration.

45. How do you measure viscosity in a high-throughput manner to feed into the DLS calculations?

Solvent viscosities and thermal models are incorporated into DYNAMICS and ASTRA software. In some cases (high concentration, buffers with certain excipients, etc.) these values will be inaccurate and must be determined empirically. This can be done in a multi-well plate format with the DynaPro Plate Reader II. Please contact Wyatt Technology for more information and protocol.

46. On slide 10 is the DLS one or multi-angle?

Our instruments, like almost all commercially available instruments, utilize single-angle DLS. 'Multi-angle' and 'MALS' refer to static light scattering, not dynamic light scattering.

47. With online DLS how is the correlation time optimized as species come of the column?

This is not generally a parameter that is optimized independently due to the rapid transit time. We set ~ 1 second acquisitions.

48. Diffusion is related to gamma/squared scattering vector magnitude. If you have a distribution within the sample the size does depend on the angle you're looking at. Please comment.

This question refers to DLS analysis and how the measurement depends on angle. To state this more clearly, the apparent distribution of DLS autocorrelation times depends on the angle at which the DLS detector is placed. Two factors affect this: the Γ/q^2 dependence of the correlation time, where q includes the sine of the angle; and anisotropic scattering intensity related to the size(s) of the particle(s), where the angular dependence is related to the rms radius R_g.

If, for example, the sample includes 'small' particles (isotropic scatterers, $R_g < 10$ nm) and 'large' particles (anisotropic scatterers, $R_g > 10$ nm), the ratio of scattering

intensity between the 'small' and 'large' ensembles depends on the viewing angle. How does DLS resolve this size distribution in a batch measurement?

First, the **values** of the sizes determined by DLS will not be skewed by the angle; since the angle of the detector is known, it is included in the calculation of size (R_h) from autocorrelation time. In order to analyze the example mentioned above for **distributions**, one *could* assume a relationship between R_g and R_h and correct for the angular intensity distribution of the light scattered by different sizes, take into account how the autocorrelation functions are weighted per scattering intensity, and so figure out the distribution of sizes independently of angle (assuming the assumption is correct).

However, the size distributions obtained from batch DLS are coarse, and the relationship between R_g and R_h unreliable, so DLS *cannot* be counted on to provide accurate or reliable measures of **quantity** or **concentration** of particles of different size, only rough indications of the presence of particles of different sizes. For an accurate analysis of distributions, a fractionation technique such as FFF is necessary.

Instrumentation and Other General Questions

49. Can a single MALS detector be used for SEC-MALS, FFF-MALS and CG-MALS? If we already have a SEC-MALS system. Can FFF be readily added/removed to the same scattering detector so that we can switch between the two?

The same MALS detector may be used for all three techniques. There is just the matter of swapping out the plumbing to connect to each of the upstream systems.

The Eclipse DualTec FFF system offers automatic switching between SEC column and FFF channel, keeping the same HPLC system and detectors. The Eclipse AF4 offers the switching feature as an option. For CG-MALS, the reconnection is simpler, and done manually.

50. Can you couple a Waters e2695 HPLC pump (and other pumps)?

The Eclipse can be integrated with HPLC lines from different manufacturers. The Eclipse control is embedded in ChemStation and in Chromeleon. HPLC instruments from Agilent and Thermo-Dionex can be used, as well as any HPLC instrument for which a Chromeleon driver is available. Integration to other leading HPLC platforms is in preparation.

51. Do you have a database of RIs for commonly used chromatography solvents?

This is included in the software.