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APPLICATION NOTE

AN2005: Characterizing size distributions of broadly heterogeneous proteins with FFF-MALS

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Introduction

Although gelatin provides important advantages for colloidal drug carriers, like its proteinaceous structure and its biodegradability, there is one major drawback of gelatin that disturbs the manufacturing process. As a consequence of the extraction of gelatin from collagen originating from different animal sources, its molecular weight distribution is usually very heterogeneous. Generating gelatin nanoparticles via desolvation is not possible when the material is broadly heterogeneous.

To overcome this problem a two-step desolvation technique was proposed and successfully realized. Part of this improved gelatin manufacturing process is the separation of the gelatin base material into high-molecular weight (HMW) and a low-molecular-weight (LMW) fractions, whereby the HMW fraction is further applied for the production of the gelatin nanoparticles. Determination of molecular weight by field-flow fractionation coupled to multi-angle light scattering (FFF-MALS) was critical to optimizing the final nanoparticles.

Materials and Methods

To determine the molecular weight and molecular weight distribution of the HMW fraction and to understand what had been empirically found for the two-step desolvation process, we used an Eclipse™ FFF system with a DAWN® MALS instrument. The Eclipse—controlled by VISION™ software—serves to separate the gelatin molecules by size; MALS data from the DAWN, combined with a UV absorbance data, provides the molecular weight and size of each eluting fraction. ASTRA® light scattering software is called by VISION to control the DAWN and record data, then calculates the results and converts the fractionation data to molecular weight moments and distributions.

We investigated the gelatin base material we usually purchased, the HMW fraction obtained during the two-step desolvation, and some modified gelatin batches from a new supplier. The concentration of all gelatin batches was 2.5 mg/mL, and 100 µL of solution were injected for each fractionation run.

Results and Discussion

During our experiments we were able to show the differences between the gelatin base material and the HMW fraction, influencing the nanoparticle's formation process (Figure 1). Furthermore, while describing the new modified batches, we were able to define a specific molecular weight and molecular weight distribution response that is necessary to simplify the nanoparticle formation to a one-step desolvation process (Figure 2).

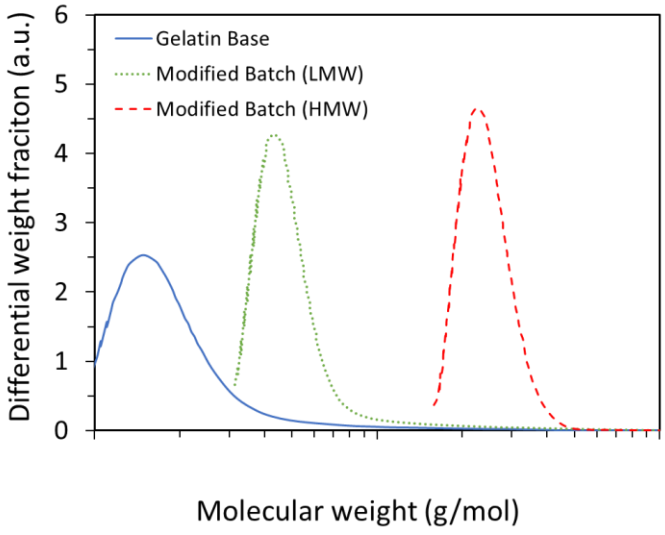

Figure 1. The differences in the molecular weight distribution between the gelatin base and the new HMW fraction are shown. Even the slightly shifted balance point of the new material enables us to produce gelatin nanoparticles by means of a one-step desolvation procedure.

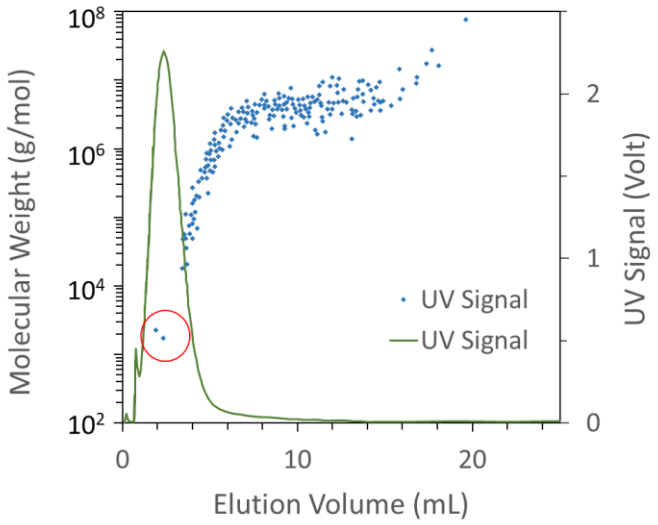

Figure 2. Molecular weight of one of the new experimentally modified gelatin batches (blue squares), overlaid on the UV fractogram (source data for Figure 1, green line). The graph shows the reduced amount of the LMW fraction of this experimentally modified batch (red circle)

Conclusion

Effective separation of HMW protein species, well beyond the capabilities of size-exclusion chromatography, is afforded by an Eclipse-based FFF system. Coupling MALS and UV detectors to FFF enables molecular weight measurements of such species across many orders of magnitude through tens of millions of g/mol. These capabilities are essential for developing protein-based drug delivery nanoparticles.

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AN2613: Analysis and characterization of fluorescent nanomaterials by FFF-MALS-FD

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Summary

Recent reports by the FDA and the European Union acknowledge the lack of robust characterization methods for nanomaterials as a major limiting factor to the final establishment of nanotechnologies. We present here results indicating that field-flow fractionation (FFF) with online multi-angle light scattering (MALS) and fluorescence detection (FD) can become an unparalleled technique for the analysis and optical characterization of novel structured fluorescent nanomaterials.

Introduction

This study was performed on silica nanoparticles (SiO_2 NPs) doped with oligothiophene (TFs). TFs are molecular dyes characterized by high chemical and optical stability and bright emission over the entire visible range. Two main aspects to be considered to optimize the synthesis of TF-SiO_2 NPs: a) NP aggregation, which may affect NP diffusivity and thus, the ability of NPs to permeate cell membranes, and b) actual inclusion and self-organization of the dyes inside the NPs, which determine the spectroscopic properties of NPs. FFF-MALS separates the particles by size and determines the size of each eluting fraction. Online fluorescence detection together with size measurements identifies free TFs versus TF-doped NPs and highlights the differences in their spectra.

Materials and Methods

We determined the NP aggregation state exploiting the unique capabilities of an Eclipse™ FFF System with a DAWN® online MALS detector to size-separate and size-characterize NPs in liquid dispersion, where MALS provides the rms radius R_g. A WyattQELS™ dynamic light scattering (DLS) module was embedded in the DAWN for determination of hydrodynamic radius R_h. MALS and DLS data were analyzed with ASTRA® light scattering software.

Experimental conditions for size determination of the silica NPs (Figure 1) were: FFF: channel dimensions = 24 cm x 21.5 mm x 350 µm (length x width x thickness); membrane = regenerated cellulose, MWCO 10 kDa; mobile phase = 5 mM tris buffer (pH 8.7); detector flow = 1.0 mL/min. Experimental conditions for fluorescence measurements (Figure 2) were: FFF: channel dimensions = 24 cm x 21.5 mm x 250 µm (length x width x thickness); membrane = regenerated cellulose, MWCO 10 kDa; mobile phase = 50:50% v/v EtOH/H_2O; detector flow = 0.65 mL/min, crossflow = 0.15 mL/min, fluorescence excitation wavelength: λ_{ex} = 325 nm.

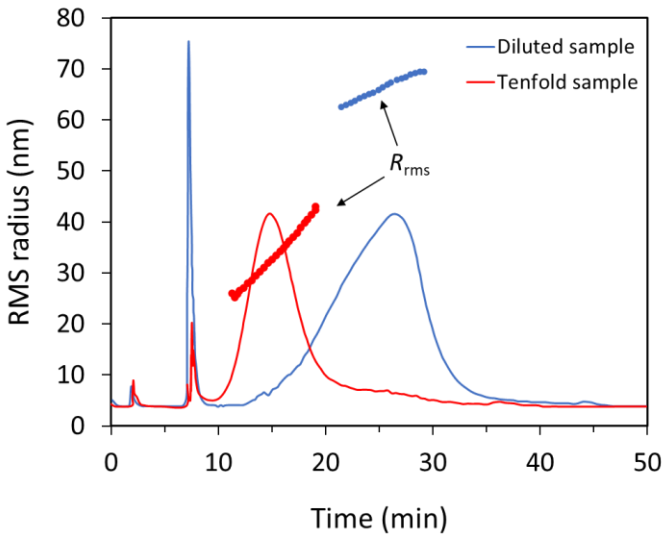

Figure 1. FFF-MALS fractograms of oligothiophene-doped silica NPs injected at different concentrations. Diluted sample injection volume: 200 µL.

Results and Discussion

Figure 1 reports the results obtained by injecting TF-SiO_2 NP samples at different concentrations. The 'tenfold' sample was 10 times more concentrated than the 'diluted' sample. R_h (not shown), R_{rms}, and R_{rms}/R_h values (not shown) suggest that the diluted sample contained mostly single, spherical TF-SiO_2 NPs. Non-spherical aggregates were found for the more concentrated sample.

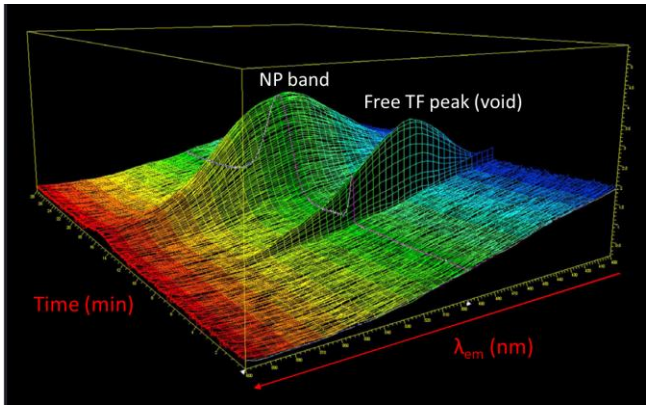

Figure 2. Fluorescence emission intensity as a function of emission wavelength and retention time of SiO2 NPs including blue and green TFs.

Spectroscopic properties of TF-SiO_2 NPs were assessed by FFF with on-line fluorescence detection. AF4 was employed to separate TF-SiO_2 NPs from non-included TFs. In Figure 2 the fluorescence intensity as a function of the emission wavelength and retention time is reported for SiO_2 NPs incorporating two different TFs. The shift in the NP emission spectrum, due to the occurrence of Förster resonance energy transfer (FRET) between the different TFs trapped in the same NPs, unambiguously proves the purity of TF-SiO_2 NPs upon fractionation with Eclipse.

Conclusions

Structured fluorescent nanomaterials are slated to become valuable medical diagnostic tools and therefore require robust characterization methods. FFF-MALS-FD, providing size-based separation followed by different structural and chemical analytics, is shown to constitute a key component in the characterization toolbox for these NPs. FFF is an automated separation method that makes use of HPLC components like autosamplers and pumps, while offering 21 CFR Part 11 – compliant software, making it eminently suitable for the requirements of producers of medical diagnostic NPs.

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AN2614: Virus-like particle characterization using FFF-MALS

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Introduction

Virus-like particles (VLP), used for vaccination and immune stimulation, are of growing interest in the pharmaceutical sciences. In quality assurance there is a great need for techniques that characterize different VLP fractions (fragments, monomers, dimers, trimers, and aggregates). We have recently demonstrated that the separation and subsequent quantification of different VLP species is possible by field-flow fractionation combined with multi-angle light scattering (FFF-MALS). Common disadvantages of this technique, like long equilibration and analysis times, the need for high sample amounts and large eluent volumes, are overcome through the use of short channel geometries.

Materials and Methods

A stressed VLP sample was analyzed by an FFF-MALS system comprising an Eclipse™ FFF controller, standard HPLC modules such as pump, autosampler and online UV detector, and a DAWN® MALS detector. Either a Short Channel or a Long Channel was used, both with a 350 µm spacer height. MALS and UV data were analyzed to determine molar masses of the eluting fractions in order to assign identity of monomer, fragment, dimer, trimer or higher aggregate.

Results and Discussion

Comparative AF4 measurements of VLPs with the Long and Short Channels revealed similar peak heights when 20 µg of VLP were injected in the Long Channel vs. 10 µg VLP in the Short Channel (Figure 1). The increased peak heights obtained with the Short Channel for low sample injected masses are due to sharper peaks and the resolution is slightly better. At the same time, analysis duration and solvent volume were reduced significantly (Table 1).

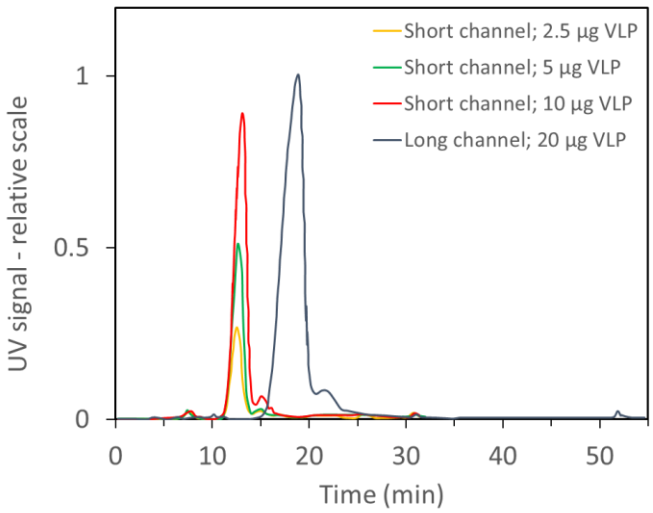

Figure 1. Comparison of peak heights, and therefore sensitivity, between Long and Short Channels.

Table 1. Comparison of duration, eluent volume and sample mass between the Long and Short Channels for equivalent sensitivity.

	Long channel	Short channel
Time/run	56 minutes	31 minutes
Eluent volume/run	159 mL	70 mL
Injection Amount	20 µg	2.5-10 µg

Using the Short Channel, analysis is possible with significantly less sample amount. In Figure 2 we see close correspondence, repeatability and accuracy of the elution times and molar masses of the various species despite quite different injected masses.

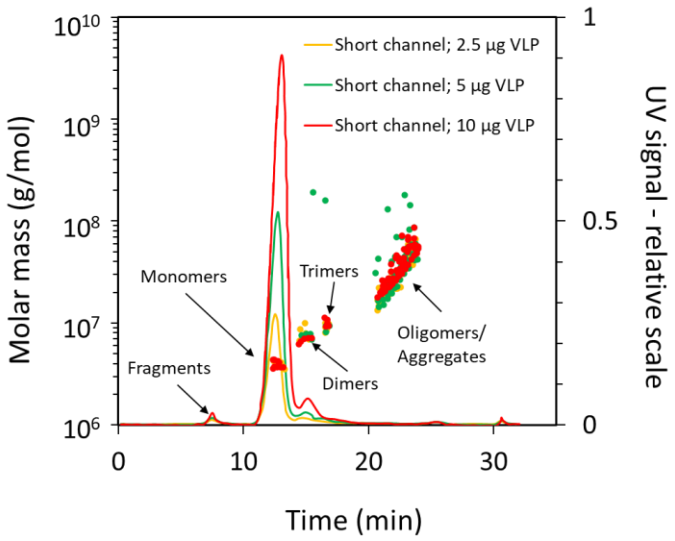

Figure 2. Different injection amounts compared, all using the Short Channel.

Conclusion

Traditional FFF long channels have limitations concerning sample amount and separation time. By contrast, utilizing Wyatt's Short Channel, analysis of far lower VLP amounts is possible in clearly shorter time and remarkably lower eluent volumes. Thus, it can be stated that the Short Channel is a clear improvement for VLP characterization as compared to the Long Channel.

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