Objective characterization of liposomal drug delivery platforms: Using cryoTEM and designated image analysis software

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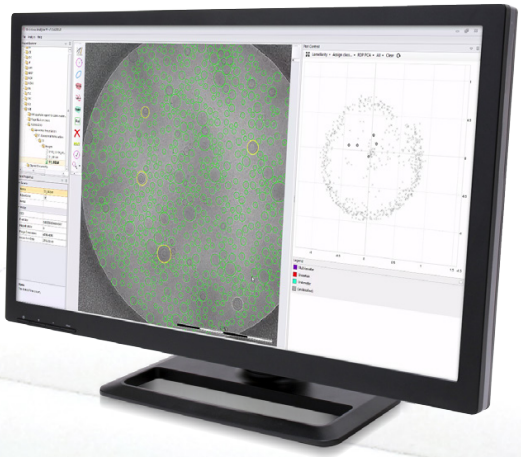
Quality-critical attributes particular to liposome drug products include physicochemical properties such as particle size distribution and morphology, e.g. their overall shape. Such properties will influence the biological activity and bio-distribution of liposomal-based drug and gene delivery platforms. Analytical techniques that provide objective and reliable information on liposome particle characteristics can thus help to improve product development processes and shorten the time to securing their final quality.

Cryo-transmission electron microscopy (cryoTEM) combined with image analysis using proprietary Vironova Analyzing Software (VAS) represents an optimized methodology for achieving this goal. By enabling semi-automated particle detection and classification analysis, statistically significant results are delivered in a time and cost-effective manner.

The study described here presents a typical liposomal characterization achieved by extracting relevant data of images obtained from cryoTEM using Vironova Analyzing Software. It includes analyses of size distribution and morphology, circularity, level of packaging/state of encapsulated drug, lamellarity and lipid bilayer thickness.



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Introduction

Liposomes are nanovesicles composed of a bilayer formed by amphiphilic molecules such as phospholipids that enclose a central aqueous compartment. Apart from unilamellar liposomes, i.e. composed of single lipid bilayers, arrangements comprising a concentric series of multiple bilayers (so-called multi-lamellar liposomes) can also be found. Typical particle sizes are generally in the range 30 to 120 nm. Such nano-sized particles can be difficult to measure and characterize accurately.

Vironova Analyzing Software (VAS) is designed to analyze transmission electron microscopy (TEM) images of nano-sized particles. By enabling reproducible and semi-automated particle detection and classification, it offers a valuable, time-saving tool for characterizing liposome drug products (Fig. 1.).

In particular, VAS semi-automates the time-consuming tasks associated with manual particle measurements that often lead to inconsistent results due to subjectivity and human error. Significant amounts of time are thus saved and the results are more consistent and reproducible.

The software works by algorithmically identifying particle characteristics based on selected criteria, such as the size or shape of the particle. It detects a large number of particles in order to reach a statistically significant number for the analysis (typically 1500 per sample).

Based on expert criteria in the form of user input, VAS supports the semi-automated classification of particles using quantitative morphology features. This expert/software combination permits complicated classifications such as packaging level/state of encapsulated drug and lamellarity to be performed in a repeatable and time-efficient manner.

Moreover, the software-enabled analysis result is presented in a report that shows the particle data as histograms and the class distribution as a bar chart. Microscopy images of the examined particles and statistical measures such as mean and median values, variance and percentiles, as well as comprehensive statistical significance and homogeneity measurements, are also clearly shown.

Sample preparation

The liposomal specimen is prepared using cryoTEM, which involves flash-freezing the sample down to cryogenic temperatures within milliseconds. This embeds the sample in vitreous ice yet has only a minimal influence on it, allowing its observation in the hydrated state. To ensure that the acquired data are representative of the sample, a large portion of the specimen area is explored and images for analysis are acquired at multiple locations on the specimen support.

Vironova Analyzer Software (VAS)

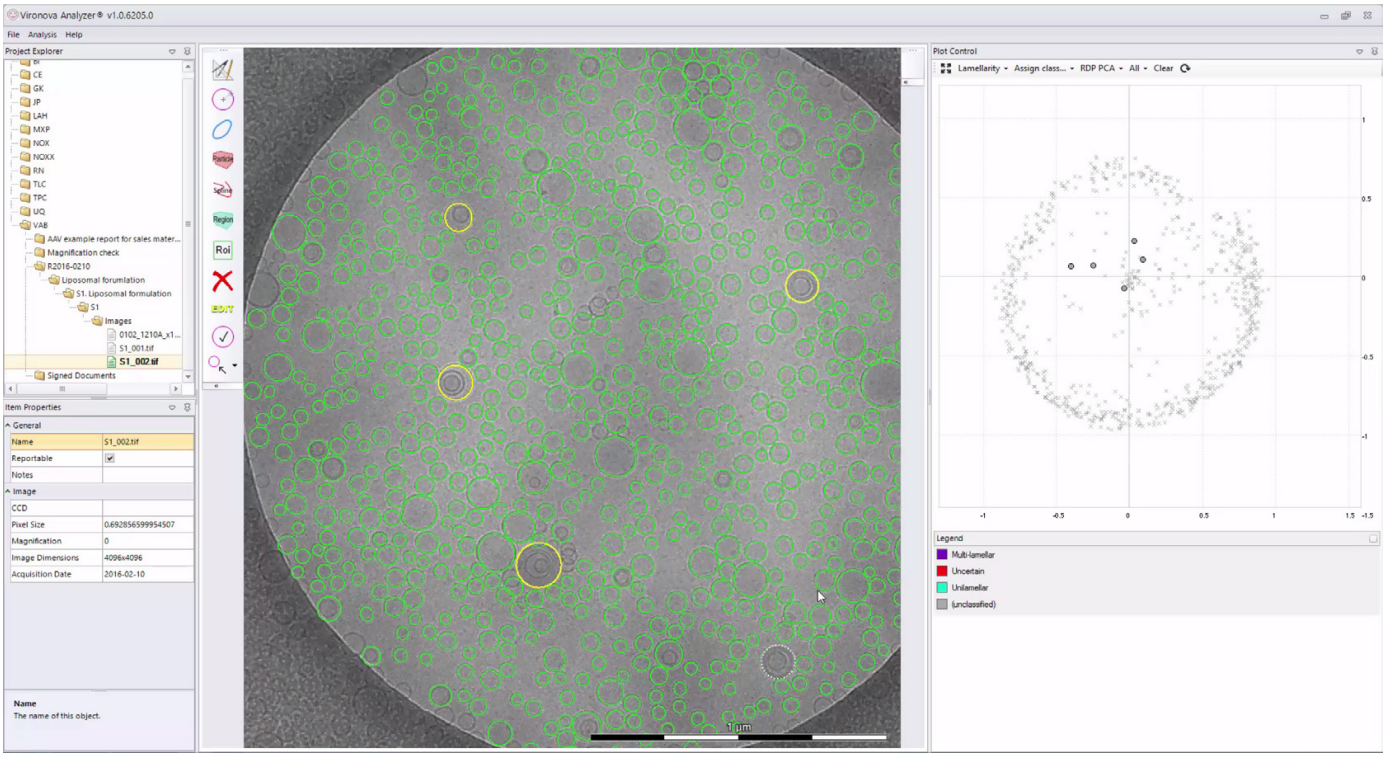

Fig 1: Vironova Analyzer Software (VAS) automatically extracts morphological data and metrics into graph-plotting and report-generating tools. Using VAS for automated nanoparticle detection can save up to 6 hours per sample compared to manual approaches.

Results

Figure 2 shows liposomes packaged with doxorubicin. The image shows particles of varying size and shape; some are spherical while others are more elongated. These variations may be responsible for differences in the therapeutic performance and bioavailability of the encapsulated drug. To assess the degree of dispersity in terms of size and circularity, these parameters were monitored, measured and presented as a scatter plot showing a distribution of small spherical versus enlarged elongated particles.

Stability study: particle size and shape analysis

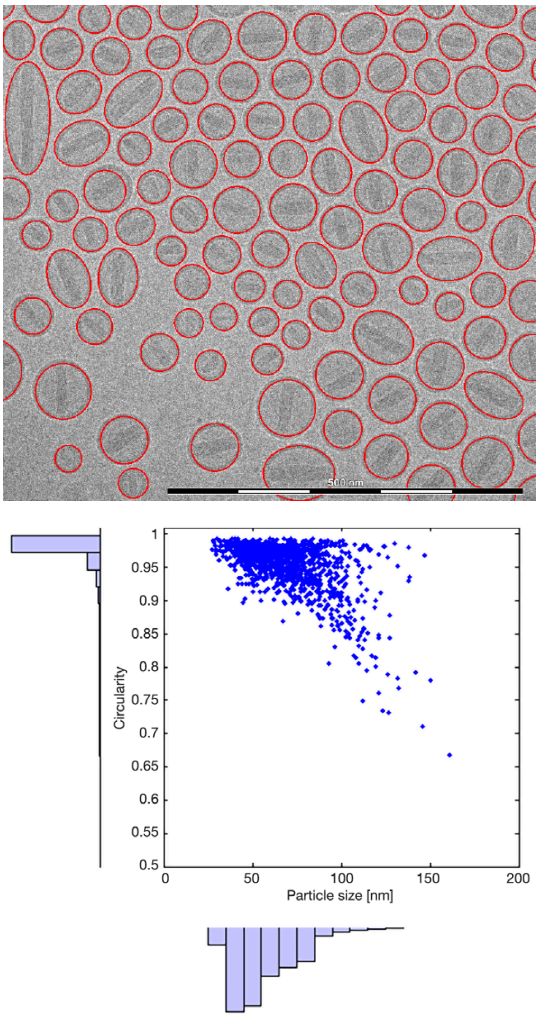

Fig 2: Upper: CryoTEM image of detected liposomes containing doxorubicin. Lower: Particle size distribution correlated to circularity at one point in time.

The degree of encapsulation is one of the key parameters that needs to be assessed and controlled during the drug development process. In this study, liposomes packed with doxorubicin crystals were detected and classified based on electron density visualized as intensities of gray to identify filled versus empty particles. Results of the particle classification are presented as a diagram (Fig. 3). Particles that could not unambiguously be classified as either empty or filled are presented as uncertain.

Filled vs. empty analysis

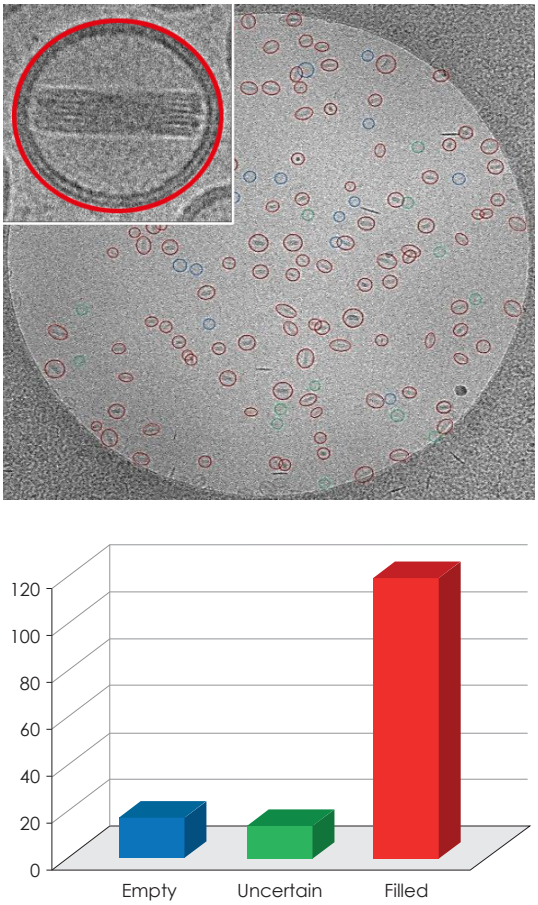

Fig 3: Upper: CryoTEM image of detected liposomes containing doxorubicin. Lower: Particles classified into three categories.

The resolving power of cryoTEM imaging also makes it possible to accurately observe intra-liposomal structures such as the level of lamellarity. This information may be critical when encapsulating certain drugs as it directly influences bioavailability. In the present study, image analysis revealed the liposome sample to be a mix of unilamellar and multi-lamellar liposomes (Fig. 4).

Morphology analysis: level of lamellarity

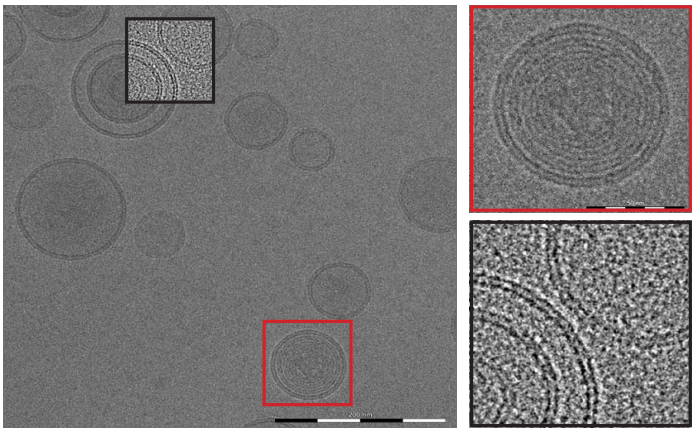

Fig 4: Left: CryoTEM image of a mix of liposomes of different lamellarities. Right: Close up of the liposomal membranes in which the resolution clearly distinguishes the lipid bilayer.

In some cases, regulations require measurement of the liposome lipid bilayer. The resolving power of cryoTEM, in combination with VAS, allows reproducible and accurate data to be extracted from the obtained images. Figure 5 presents a case study where the membrane thickness of the liposomal bilayer is accurately measured by estimating differences in the relative electron density, appearing on the image as intensities of gray. In this case, it correlates to a thickness of 5 nm.

Morphology analysis: thickness of the liposome lipid bilayer

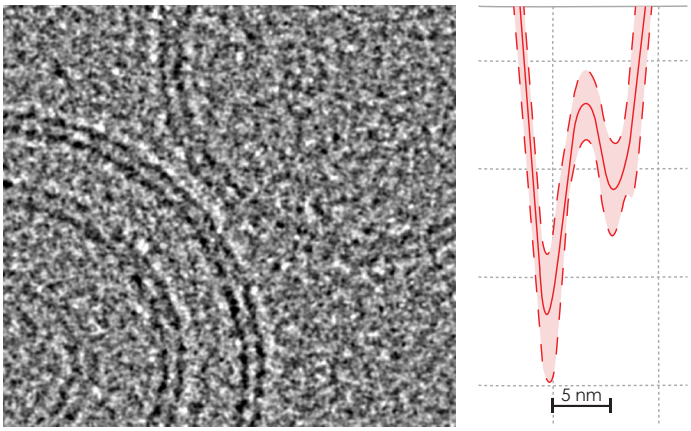

Fig 5: Left: CryoTEM image of a detail of the lipid bilayer. Right: Graphical representation of the lipid membrane thickness, 5 nm in the present case.

Conclusions

The physicochemical properties of liposomal drug products frequently require accurate measurement and characterization. Aspects of particular interest include the morphology of the liposome (including lamellarity determination), their structure and integrity (including changes due to different preparation or storage conditions), encapsulation efficiency (filled/empty) and particle size distribution. CryoTEM combined with VAS enables reliable measurement of these key parameters in an objective and statistically significant manner.

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