Cryo-TEM and Image Analysis of Polymer Nanoparticle Dispersions

Vanessa Durrieu¹, Jean-Luc Putaux ², Raphaël Passas¹ and Alessandro Gandini ¹

ABSTRACT

Light scattering is routinely used to measure the average particle size in aqueous polymer dispersions. However, difficulties arise for polydispersed populations, nanometric particles, absorbent materials or mixtures of materials with different densities. A complementary approach consists in combining cryo-TEM and image analysis. Using these techniques, we studied various polyurethane aqueous dispersions. The nanoparticles were characterised in terms of morphology and size. Average diameters, calculated using a semi-automatic image analysis procedure, were compared to those obtained from dynamic light scattering (DLS) experiments.

MATERIALS AND METHODS

Aqueous polymer dispersions

Polyurethane-urea dispersions were prepared according to the method described elsewhere [6,7]. A polyurethane pre-polymer with reactive isocyanate chain ends was synthesised and dispersed in water. Chain extension was carried out and nanoparticles formed. Several dispersions were studied, varying the nature and proportion of the raw materials (di-isocyanates, polyols and emulsifying agents).

Light scattering

DLS was carried out using a Malvern Autosizer 2c photon correlation spectrometer (Malvern Instruments, Orsay, France) equipped with a HeNe laser (10 mW, 632.8 nm) and operated at an angle of 90˚. The Z-average mean diameter \( D_{Z(DLS)} \) was calculated using the cumulant analysis (standard method ISO 13321). Dispersions exhibiting a polydispersity index (as defined by the spectrometer) higher than 0.25 were discarded from our comparative study.

Cryoelectron microscopy

According to the method described elsewhere [6,7], thin liquid films of 1 mg/ml nanoparticle dispersions were prepared onto ‘lacey’ carbon films (Pelco, USA) and quench-frozen into liquefied ethane (Fig 1). Once transferred into a Gatan 626 cryoholder, the specimens were cryo-electron microscope operated at 80 kV. Micrographs were recorded on Kodak SO163 films at magnifications of 11,500 and 20,000, with a defocus of 1.2 µm.

Image analysis and size distribution

The negatives were digitised using a Kodak Megaplus CCD camera. The magnification was chosen in order to include a large number of particles in the field of view, while maintaining a sufficient resolution. A sampling rate of 2 mm/pixel was considered to be a good compromise. Contrast enhancement and semi-automatic particle measurements were performed using routines from the Optimas 6.51 software (Media Cybernetics, Inc.) [8]. The number-average diameter \( D_n \), the weight-average diameter \( D_w \), as well as a polydispersity index \( P_d \), were defined using the following formulas:

\[
D_n = \frac{\sum W_i D_i}{\sum W_i}, \quad D_w = \left( \frac{\sum W_i D_i^3}{\sum W_i} \right)^{1/3}, \quad P_d = \frac{D_w}{D_n}
\]

where \( W_i \) is the number of particles at the \( i \)th class in the size-distribution histogram and \( W_i \) is the weight of the \( i \)th class.

Figure 1:

(a) Production of thin vitrified films for cryo-TEM. 1. The excess of suspension was blotted with a filter paper. 2. The grid was quench-frozen into liquefied ethane at -171°C. (b) Schematic transverse view of a vitreous ice film containing polymer particles.
Mean diameters and polydispersity indexes calculated from cryo-TEM images and DLS experiments for four different polyurethane dispersions.

The contrast between the polymer particles would be insensitive to this type of morphological feature. This is a typical example where the direct visualisation of the dispersion provided information about the mechanism of particle formation, whereas DLS was insensitive to this type of morphological feature.

Image analysis

The contrast between the polymer particles and the embedding ice was generally low in cryo-TEM images, partly due to the strong inelastic electron scattering. Contrast enhancements were performed but they also increased the contribution of the background noise from the negatives. On the other hand, variations in the ice thickness induced background intensity modulations. Consequently, the grey-level histogram of the cryo-TEM micrographs generally exhibited a broad peak (Fig 3a) and it was difficult to define luminance classes corresponding to specific contributions from the supporting membrane, the particles and the embedding medium. To limit these problems, the treatments were performed on smaller regions of interest (ROI) selected by the user (Fig 3b). To get a better contrast, the TEM micrographs were recorded with a negative defocus, generating fresnel fringes around the ice-embedded objects. Considering the high background noise, only a white fringe located outside of the particle surface could be seen. However, as we mostly used 1 to 2 nm defoci, we assumed that fresnel effects would not significantly influence the particle detection.

The main steps of the image processing procedure were:

1. Manual selection of a rectangular ROI (Fig 3b, step 1).
2. Local adaptive background smoothing to homogenise the luminance and improve object detection: this routine fits a polynomial surface to the sampled background and corrects the ROI luminance by subtracting this surface [8].
3. Manual thresholding based on a visual control, followed by an image binarisation (Fig 3b, step 2).
4. Elimination of background heterogeneities using successive erosions/dilatations.
5. Edge detection of the particles (Fig 3b, step 3) and separation process.

In addition, due to surface tension effects, the frozen film often exhibits a gradient of thickness. Consequently, the average background intensity in the image varies over the field of view. As explained earlier, binarisation can only be properly carried out after background fitting and correction. Another consequence is the fast reorganisation of the particles in the liquid film, just before freezing. The largest particles tend to accumulate near the centre of the film, while the smaller ones remain closer to the centre of the film. Depending on the local concentration and steepness of the gradient, regular distributions may be observed, the nanospheres reorganising along iso-thickness lines according to their size (Fig 5b). However, we cannot rule out the fact that some bigger objects may have migrated towards areas that are too thick for proper observation. These 'hidden' particles would thus be absent from the statistical treatment, which would yield an error in the mean diameter calculation. When the film

Table 1:

<table>
<thead>
<tr>
<th>$P_o$</th>
<th>$D_{10}$ nm</th>
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In order to validate our semi-automatic procedure, we checked that 'manual' measurements performed on the same images and numbers of particles yielded similar results. However, as expected, the semi-automatic image analysis generally ran four times faster. An example or size distribution histogram is presented in Fig 4b.

Surface tension effects may influence the particle distribution during sample preparation. Controlling the thickness of the vitreous ice film is generally difficult and depends on the diluent composition (salts, surfactants, organic solvents), the room humidity level, the blotting efficiency, etc. Thickness values that are proper for TEM imaging typically vary from 50 to 200 nm [7]. The presence of a single layer of particles in the film is important, as illustrated in Fig 5a: the larger nanospheres are more or less isolated, while the smaller ones overlap along the observation axis. In that case, size-distribution analysis is hardly possible, although the operator still has a good general impression on the suspension dispersion.

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thickness is more or less constant, particles can mix more randomly, at the risk of overlapping (Fig 5a).

With a $T_g$ lower than room temperature, the suspended nanoparticles are soft in the liquid film and thus sensitive to the surface tension effects. If their size in the dispersion is larger than the local thickness of the liquid film, and provided that they cannot ‘migrate’ to thicker areas, the nanoparticles are squeezed within the thin film. Their apparent diameter thus increases, yielding an incorrect spread of the size-distribution histogram towards larger diameters. Indeed, it is difficult to visualise such an effect and judge if the particles are actually deformed. In our experiments, we assumed that the particle size might be unreliable above 100-120 nm.

After binarisation, the image analysis algorithm performs several erosions/dilatations which, depending on the particle shape, may generate an error on the contours. This error was estimated to be around 1 pixel, i.e. 2 nm, considering our sampling rate. Moreover, the successive erosions, carried out during the image ‘cleaning’, eliminated objects smaller than 5-6 pixels. Consequently, particles smaller than 10 nm were not properly taken into account in our size-distribution histograms. This is readily visible in Fig 4b although the effect was not detected in most samples.

Finally, several remarks can be made when comparing the mean diameters obtained by DLS and image analysis. Values corresponding to four different dispersions with increasing polydispersity index $P_d$ are given in Table 1. First of all, there is a significant difference between the values of $D_{Z(DLS)}$ and $D_{Z(TEM)}$ which increases at higher polydispersity. This could be expected, since the detection is based on different properties of the particles. In particular, DLS gives more statistical ‘weight’ to the larger particles which are more highly scattering objects. Moreover, DLS is sensitive to the ionic environment of the nanospheres that cryo-TEM cannot visualise since it has the same density as the embedding medium. The comparison of $D_{Z(TEM)}$ (calculated from the cryo-TEM data as a D(6,5) statistical mean) with $D_{Z(DLS)}$ is more relevant. $D_{Z(TEM)}$ always appears to be larger than $D_{Z(DLS)}$. The values are close when $P_d$ is low, but the deviation becomes larger with increasing polydispersity. The fourth case, with $P_d = 1.50$, perfectly illustrates how cautious one has to be to interpret and compare the data obtained with techniques sensitive to different properties of the particles.

**CONCLUSION**

In this work, several techniques were used concurrently to study aqueous dispersions of polyurethane nanoparticles. Light scattering was non-destructive and allowed us to characterise rapidly a large number of samples over a wide range of sizes. However, it showed some limitations in the case of polydispersed distributions and with particles smaller than 50 nm. The combined use of cryo-TEM and image analysis appeared to be complementary to DLS. In particular, the absence of aggregation as well as differences in nanoparticle morphology were important results provided by this direct visualisation. However, the size analysis of soft polymer particles only made sense for diameters below 100 nm. Moreover, the smaller objects (<10 nm) may not have been properly detected. These limits would obviously vary for different polymers, depending on factors such as particle density, crystallinity and $T_g$. In addition, the use of zero-loss energy-filtered imaging would certainly increase the contrast of cryo-TEM micrographs and consequently improve particle detection.

**REFERENCES**