TEM analysis of nanoparticle dispersions with application towards the quantification of \textit{in vitro} cellular uptake

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\textbf{Abstract.} TEM analysis of nanoparticle dispersions in solution has previously been limited, in the main, to the measurement of primary particle size due to the drying effects that occur during sample preparation. We show that solutions prepared for TEM by plunge freezing specimen grids coated with a blotted solution is a sensitive and potentially representative route to measuring a nanoparticle dispersion. We have started to benchmark the technique against the more commonly used dynamic light scattering method and show that the TEM route is potentially more sensitive route to counting individual nanoparticles in a dispersion that also contains nanoparticle agglomerates. Accurate measurement of nanoparticle dispersion in biological solutions could be a key step in the application of nanoparticles in medicine.

1. Introduction

Nanoparticles are finding increasing applications in the field of medicine \cite{1}, with extensive research being conducted on the design of nanoparticles to enhance such applications \cite{2}. Preliminary investigations of nanoparticles with potential applications in medicine involve \textit{in vitro} testing to explore cellular uptake and action. Transmission electron microscopy (TEM) is a valuable tool for the intracellular localization of nanoparticle uptake and for the development of mechanistic modes of action. TEM studies are often conducted on the nanoparticles prior to \textit{in vitro} delivery and subsequent to it on ultrathin sections of cells.

The use of nanoparticles in medicine is far more complicated than the use of soluble chemicals \cite{3}. Characterisation of nanoparticles prior to \textit{in vitro} delivery is affected by two factors. Firstly, nanoparticles are rarely used in an as-received or as-synthesised state; the nanoparticles are commonly dispersed in biological media which affects the surface chemistry and the agglomeration state. Secondly, the most commonly used dispersion characterization technique of dynamic light scattering (DLS), is not able to distinguish between different types of particles in the solution and more importantly becomes significantly less reliable for the measurement of wide particle size distributions (because of the sixth power dependence of light scattering intensity on the scattering particles’ sizes). DLS accuracy is also reliant on knowing the refractive index of the nanoparticles and the viscosity and refractive index of the dispersant \cite{4}. On the other hand TEM imaging of samples prepared by drop-
casting is not representative of the dispersed state of the nanoparticles owing to drying effects and can only really be used to examine the primary nanoparticles [5].

We have investigated an alternative TEM sample preparation route to produce solution derived specimens with more representative dispersions of nanoparticles. We are primarily interested in the dispersion of cadmium selenide/zinc sulphide quantum dots, on which studies regarding statistical analysis of the cellular uptake have been reported and in which DLS results have shown a heterodispersion of the dots [6]. Using sample preparation, which involves the plunge freezing of TEM grids coated with blotted solutions and a combination of cryo- and room temperature-TEM we demonstrate that this preparation route produces a specimen with a range of nanoparticle agglomerate sizes. We also discuss correlation with dynamic light scattering size profiles of the same solutions.

2. Experimental
CdTe/ZnS core/shell quantum dots with specific peptide molecules on their surface to facilitate cell entry (Qtracker 705, ~10 nm in length, Invitrogen Ltd) were dispersed in McCoys Medium 5A Modified Medium (Sigma Aldrich) at a concentration of 10 nM. Ludox SM-30 silica nanoparticles (Ludox Colloidal Silica, ~15 nm in diameter) were dispersed in Dulbecco’s Modified Eagle’s Medium (Lonz, Slough, UK) at a concentration of 100 µg/mL.

Samples for cryo-TEM were prepared by placing a 3.5 µL droplet on a glow discharge treated carbon film (R1.2/1.3 Quantifoil MicroTools GmBH), blotted and plunge frozen in liquid ethane [7].

Cryo-TEM was conducted at liquid nitrogen temperatures (77 K) on an FEI Tecnai F20 FEG-TEM operated at 200 kV and equipped with a Gatan Ultrascan 4000 CCD camera. Standard TEM was conducted on an FEI Tecnai F20 FEG-TEM operated at 200 kV and equipped with a Gatan Orius SC600A CCD camera. DLS was conducted on a Malvern Zetasizer Nano ZS with subsequent data manipulation conducted using the Zetasizer software (Version 6.20).

3. Results and Discussion
A sample of the CdSe/ZnS quantum dots (QDs) dispersed in water was prepared by plunge freezing a blotted TEM grid into liquid ethane [7] and examined by cryo-TEM. This allowed imaging of the QD dispersion through a thin film of vitrified ice, such that a range of agglomerates in the solution are visible (figure 1). A lack of QD signal to background is evident in the low magnification images and this is due to two major factors: the low electron fluence required to avoid devitrification of the ice and the variable thickness of the ice. These combine to make identification of individual or small groups of QDs difficult, and consequently skew any data collection towards the identification and counting of larger agglomerates.

To overcome these problems we heated the sample in the TEM from cryo conditions (~77 K) to a temperature (~160 K) at which the ice devitrifies and then sublimes. It is known that during devitrification the water and any macromolecules present exhibit little movement [8]. TEM imaging of the same agglomerates after sublimation of the ice show that the QDs do not move and that the same size agglomerates could be measured (figure 1 (b) and (c)). This allows for the sample to be prepared via the plunge freezing route, but then analyzed in a TEM at room temperature after freeze drying. Without the ice, a higher electron fluence can be used (the specimen is far less beam sensitive) and there is a weaker more consistent background level (from the carbon support film) and so it is much more straightforward to also identify small groups and individual dots across the grid.
Figure 1. (a) Low magnification cryo-TEM image of the vitrified solution on the support film. Agglomerates of QDs are clearly visible however it is difficult to identify individual dots. TEM images of an agglomerate (b) before and (c) after devitrification and sublimation of the ice. In order to qualitatively validate this preparation route for TEM sizing against DLS sizing, a simple system of uncoated silica nanoparticles was investigated. The silica nanoparticles, as observed by DLS, are monodisperse in water and agglomerate in biological media (figure 2 (a)). Samples of the water and biological media dispersions were prepared for TEM via the plunge freezing method. Room temperature TEM imaging of the two solutions are consistent with the DLS size profiles, monodispersed nanoparticles were visible in the water dispersion (figure 2 (b)) and agglomerates of several hundred nanometers were visible in the water plus biological media dispersion (figure 2 (c)).

Figure 2. Analysis of silica nanoparticle dispersions in water and water plus media (a) DLS number plot derived from the original intensity-based light scattering data, (b) TEM image of monodisperse silica nanoparticles in the water dispersion and (c) agglomerates in the water plus media dispersion.

For a more quantitative comparison with DLS we measured a dispersion of the QDs in water plus biological media, however this is now complicated by the peptide coating on the nanoparticle surface. Coating is common for nanoparticles with medical applications in order to target specific interactions, in this case cellular uptake by endocytosis. The coating however will also affect the dispersion of the dots in the biological media prior to uptake. The inorganic salts, amino acids, vitamins and other components of the media will affect the agglomeration of the QDs. A TEM grid containing a dispersion of QDs in biological media was prepared by plunge freezing and was imaged by TEM at room temperature. In total, 480 individual or agglomerated groups of QDs were imaged from 216 images, which were recorded by examining 17 grid squares from two separate grids. The size of each agglomerate was given by the distance between the two most spaced QDs in the cluster. To provide a comparison to the DLS results (figure 3 (a)) the TEM derived measurements were then grouped to the nearest 10 nm, and presented on a frequency plot (figure 3 (b)). The TEM results, as shown by both the size distribution plot (figure 3 (b)) and several images (figure 3 (c)-(e)), suggest that there is a fraction of agglomerates on the scale of 100 nm in size (as suggested by DLS) but that there is also a more significant number of mono-dispersed/isolated dots. This difference may be due to an artifact of the TEM preparation route or to the relative insensitivity of light scattering to the smallest fraction of the QD dispersion. The latter would be significant if individual dots were also taken up by cells during in vitro exposure. To explore the difference between the two techniques in more detail we are currently measuring simpler dispersions of QDs in water.
Figure 3. Analysis of QDs in biological media (a) DLS number plot (b) frequency plot from TEM measurements (c)-(e) TEM images of agglomerated and individual QDs.

4. Conclusions
The preparation of samples via the plunge freezing route provides a potential avenue for the analysis of the dispersion state of nanoparticles in biological fluids. It is known that when nanoparticles are dispersed in biological fluids the sizes formed are variable and dependent upon both the nanoparticles and dispersants. Other characterization techniques such as DLS are potentially insensitive to the finer fraction of a nanoparticle dispersion if the size range is wide, whereas samples prepared via plunge freezing and then analyzed by TEM do enable counting of agglomerates and individual particles.

Acknowledgements
This work has been supported by the EPSRC in Leeds (EP/E059678/1 and EP/H008578/1) and Swansea (EP/H008683/1).

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