Cryo-STEM-EDX spectroscopy for the characterisation of nanoparticles in cell culture media

IOP Conf. Series: Journal of Physics: Conf. Series **902** (2017) 012006 doi:10.1088/1742-6596/902/1/012006

Cryo-STEM-EDX spectroscopy for the characterisation of nanoparticles in cell culture media

M Ilett[1], F Bamiduro[1], O Matar[1], A Brown[1], R Brydson[1] and N Hondow[1]

[1] School of Chemical and Process Engineering, University of Leeds, Leeds LS2 9JT, UK

pmmi@leeds.ac.uk

Abstract. We present a study of barium titanate nanoparticles dispersed in cell culture media. Scanning transmission electron microscopy combined with energy dispersive X-ray spectroscopy was undertaken on samples prepared using both conventional drop casting and also plunge freezing and examination under cryogenic conditions. This showed that drying artefacts occurred during conventional sample preparation, whereby some salt components of the cell culture media accumulated around the barium titanate nanoparticles; these were removed using the cryogenic route. Importantly, the formation of a calcium and phosphorus rich coating around the barium titanate nanoparticles was retained under cryo-conditions, highlighting that significant interactions do occur between nanomaterials and biological media.

1. Introduction

The promise shown by nanomaterials within medical research remains restricted by the difficulty in fully characterising nanoparticles within complex biological media [1]. Consequently, novel approaches are required to ensure nanomaterials can be fully characterised within this complex media. Commonly, *in vitro* investigations require nanomaterials to be dispersed in cell culture media for cell uptake studies. Therefore, the need to successfully monitor the nano-bio interactions that occur between nanoparticles and complete cell culture media (CCCM) has been identified.

Here we present an investigation looking at barium titanate (BT) nanoparticles dispersed in CCCM. BT nanoparticles have shown promise for use within medical imaging, cancer therapy, drug/gene delivery and tissue engineering [2]. Accordingly, a comprehensive understanding of how the nanoparticles interact with CCCM is required to better understand the results from *in vitro* cellular exposure experiments.

Analytical transmission electron microscopy (TEM) is one of the leading techniques for nanoparticle characterisation, with energy dispersive X-ray (EDX) spectroscopy used to provide elemental analysis. EDX spectroscopy can be combined with scanning TEM (STEM) in order to provide spatially resolved elemental maps. However, analytical TEM is often limited by the requirements during sample preparation for both thin and dry samples. Here we report on STEM-EDX spectroscopy carried out on BT in CCCM suspensions via both conventional drop casting (DC) TEM and plunge freezing with examination under cryogenic (cryo-) conditions. The results indicated a clear difference between the two techniques. This confirmed that drying artefacts can heavily influence the TEM analysis of nanomaterial suspensions, and highlighted analytical cryo-TEM as a viable option to combat this. In addition a significant interaction between the BT nanoparticles and CCCM was observed.

2. Experimental

2.1. Materials

BT nanoparticles, 130 nm in diameter were prepared by a previously reported hydrothermal method [3] and extensively characterised [4]. Dulbecco's Modified Eagles Medium (DMEM) (Sigma Aldrich, 51435C-500 ml Expiry date: Sep 2017) was supplemented with 1% penicillin-streptomycin (Sigma Aldrich, Lot# 066M4791V, P4333-100 ml) and 10 % Fetal bovine serum (FBS) (Sigma Aldrich, F7524-50 ml) and is referred to as CCCM throughout this paper.

2.2 Sample preparation

BT in CCCM stock suspensions were made at a concentration of 1 mg/ml by weighing the requisite amount of BT in 10 ml CCCM and then sonicating for a minimum of ~12 h. This stock was then diluted down to 100 μg/ml with further CCCM for analytical TEM analysis. For DC TEM grid preparation a holey carbon film TEM grid was plasma cleaned to make it hydrophilic and the BT-CCCM suspension drop cast onto it and allowed to air dry.

Cryo-TEM sample preparation was carried out using a FEI Vitrobot©. A Quantifoil TEM grid (R1.2/1.3; EM Resolutions) was plasma cleaned and then held within the Vitrobot© before a 3.5 μl drop was pipetted on the grid. The grid was then blotted and plunged into liquid ethane, after which it was maintained under liquid nitrogen before being transferred to a Gatan 914 TEM cryo-holder. The Vitrobot© settings were as follows; 100 % humidity, 30 s wait time and blot force 6.

2.3 TEM imaging and characterisation

For the drop cast specimens a FEI Tecnai F20 microscope operated at 200kV and fitted with a Gatan Orius CCD camera and Oxford instruments 80 mm^2 X-max EDX detector was used. For all cryo samples a FEI Titan^3 Themis G2 operating at 300 kV TEM fitted with 4 EDX silicon drift detectors, and a Gatan One-View CCD was used. For cryo-STEM-EDX imaging, conditions were set at dwell time 25 μs and probe current of 40 pA.

3. Results and Discussion

From TEM imaging of DC BT dispersed in CCCM a significant interaction at the nano-bio interface was identified, since a clear coating had formed around the nanoparticles (Figure 1(a)).

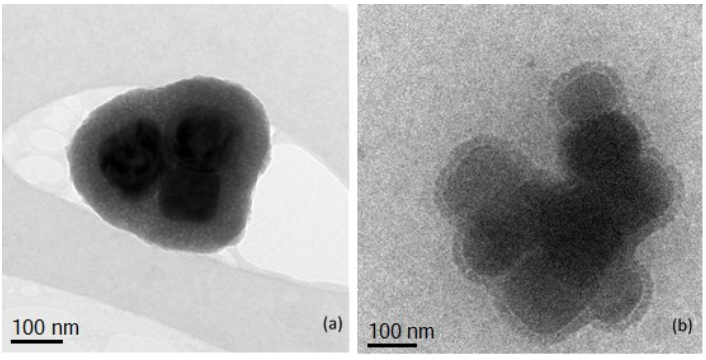

Figure 1: (a) TEM image of DC BT in CCCM showing a coating had formed around the nanoparticle agglomerates. (b) This was confirmed in cryo-TEM imaging where a similar coating was observed.

EDX analysis was carried out in order to identify the elements present in the observed coating. The EDX spectrum contained the expected Ba and Ti peaks present due to the composition of the nanoparticles themselves and a Cu signal in accordance with the use of a Cu TEM grid. In addition there were clear Ca, P, Na, Cl and Mg peaks (Figure 2). All these elements can be found within the cell culture media used which contains, inorganic salts, amino acids and vitamins, in addition to proteins from the FBS supplement. From the EDX maps, these elements appeared to be accumulating around

the BT nanoparticles forming the coating seen in the TEM images. The peaks for Ca and P were the most prominent which suggested that the coating was dominated by these two elements.

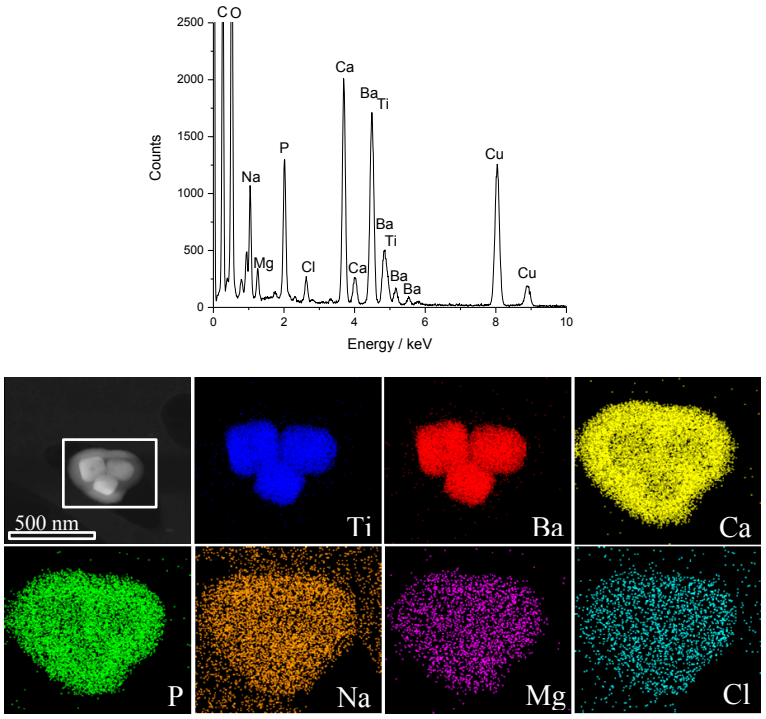

Figure 2: STEM-EDX data of DC BT in CCCM. In the EDX spectrum Ca and P are the most prominent peaks but also present were Cl, Mg and Na, all of which spatially match to the coating imaged around the BT nanoparticles.

Cryo-TEM of the suspension permits 'native' state imaging and analysis and confirmed the presence of the coating observed in TEM of the DC sample (Figure 1(b)). This suggested that it was not a result of any drying artefacts common to the DC technique but was present in suspension. Cryo-STEM-EDX analysis, carried out whilst maintaining the vitreous ice surrounding the specimen, showed a significantly different composition to the coating compared to that obtained from TEM of DC BT in CCCM (Figure 2). As expected O, Ba, Ti and Cu peaks were again present. So too were Ca and P peaks, however, the other elements observed in TEM of the DC sample (Na, Cl and Mg) were far less prominent; only a Cl peak could be confidently distinguished from the spectral background (Figure 3).

We suggest that this compositional difference in the coating can be explained by considering that during the process of plunge freezing, the nanoparticles were maintained in suspension whereas the DC sample preparation caused additional components of the CCCM to accumulate around the BT nanoparticles during drying. Thus, the cryo-STEM-EDX data suggested a coating did indeed form around the BT nanoparticles in suspension and that this was entirely Ca and P rich. This confirmed a significant interaction occurred between the BT nanoparticles and CCCM which is presumably driven by the pH of the CCCM which influences the surface charge of the nanoparticles and in turn causes interactions with certain ions in the media.

The smaller signals of Ca and P relative to Ba and Ti seen in cryo-STEM-EDX could be due to the thinner coating that was observed in cryo-TEM imaging compared to DC TEM imaging (Figure 1). However, considering the lack of any Mg and Na signals this confirmed that these elements did not adsorb to the nanoparticle surface. Although there was still a signal in the cryo-EDX spectrum for Cl, when looking at the EDX map, a far more dispersed distribution of Cl across the analysed area was seen compared to the EDX Cl map from DC TEM (Figure 2). This was indicative of the element simply being present in suspension and not as part of the observed coating.

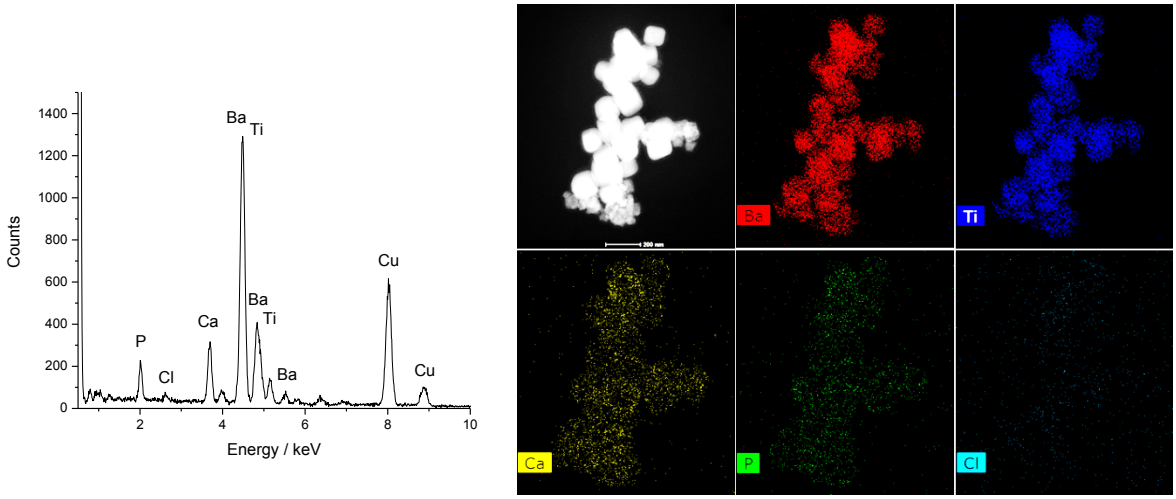

Figure 3: Cryo-EDX spectrum and corresponding EDX maps for BT dispersed in CCCM. Ca and P are present in the spectrum but through cryo analysis no visible Na and Mg peaks were present and the Cl peak was only just distinguishable. Analysis was carried out using a probe current of 40 pA.

Considering the importance of Ca and P in cell function it is likely that the accumulation of these elements around BT nanoparticles in CCCM will significantly influence the uptake and impact of the nanoparticles during *in vitro* cellular exposure carried out using DMEM. In view of this, further concerns arise due to the variable composition of commercial cell culture media; for example Roswell Park Memorial Institute, another common culture, contains roughly six times as much phosphate in comparison to DMEM [5]. Thus it would be expected that the behaviour of a nanoparticle system within one cell culture media may not be the same as within another.

4. Conclusion

A significant variation between STEM-EDX spectroscopy carried out on DC and plunge frozen samples of BT nanoparticles in CCCM established that drying artefacts in DC TEM can dramatically alter the perceived nano-bio interactions. Cryo-TEM analysis was successfully carried out to enable identification and elimination of detrimental drying artefacts to reveal a significant Ca and P rich coating formed around BT nanoparticles when dispersed in CCCM.

The study has identified the pressing need to continue the development of analytical cryo-TEM in order to more accurately characterise nanoparticles within complex media and confirmed cryo-STEM-EDX as one way to do this. Future work will look to monitor the nano-bio interactions of other nanoparticles systems within CCCM and continue to develop the possibilities within cryo-analytical TEM whereby cryo-EELS and low dose imaging can be performed.

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