Author's response to reviews

Title: An in vitro assessment of panel of engineered nanomaterials using a human renal cell line: Cytotoxicity, pro-inflammatory response, oxidative stress and genotoxicity

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Author's response to reviews: see over
Dear Editor,

Please find attached revised manuscript entitled 'An in vitro assessment of panel of engineered nanomaterials using a human renal cell line: Cytotoxicity, pro-inflammatory response, oxidative stress and genotoxicity' by Kermanizadeh et al. This manuscript is part of a larger European project FP7 which investigates the toxicology of a panel of nanomaterials in a wide variety of targets in order to relate the physicochemical characteristics to the potential hazard. This particular paper focuses on in vitro hazard to the kidneys.

We would be grateful if you would consider the manuscript for publication and please do not hesitate to contact me should you require further information.

Thank you

Kindest regards

Ali
Response to Reviewers

Reviewer 1 – Enrico Bergamaschi

Major revisions

1. Discussion has been significantly modified

2. All figures have been re-plotted and labelled

3. We thank the reviewer for their comments however the aspect ratio of the ZnO NMs are not significantly different and so this is not likely to be very important with regards to the adverse effects noted in this study especially as the ZnO materials are known to be highly soluble [14]. We suggest that the adverse effects seen in study are at least in part associated with the release of Zn ions. The following has been added to the main text:

We had previously shown that the two ZnO NMs used in this study are highly soluble (40-50%) while less than 1% of Ag (NM 300) was soluble after 24 hr of incubation in complete medium [14]. Therefore there is a real possibility that the high cytotoxicity of the two ZnO materials are in part due to the release of ions, with this scenario being less likely following exposure to the Ag NMs.

4. The TiO$_2$ NMs were chosen by the ENPRA consortium to incorporate different charge, crystal structure and size. We have expanded on our discussion section to explain the potential effects of positively charged NMs. Please see below:

We show a significant increase in genotoxicity following a 4 hr exposure to two of the five TiO$_2$ NMs investigated in this study (NRCWE 002 and NRCWE 004 - most notable following exposure to the positively charged TiO$_2$ NMs). One possible explanation for this could be that the positively charged NMs enter cells utilising faster and more effective pathways (fast attachment to cell membrane and ingestion) than their neutral and negative counterparts [39, 40, 41]. Overall our findings are similar to a recent study in which exposure of Cos-1 monkey kidney fibroblasts to TiO$_2$ NMs resulted in significant DNA damage as measured via the comet assay [42].
5. The final paragraph has been modified:

We found that the MWCNTs tested were relatively non-toxic to the HK-2 cells at the times and concentrations tested. The toxicity of MWCNTs is widely documented, with adverse effects observed in pulmonary [34], hepatic [14], renal [43], dermal cells [44] as well as monocyte [45] and macrophage cells [46]. It has been shown that exposure of NRK-52E cells (an *in vitro* renal model) to MWCNT resulted in low toxicity (LC25 following 24 hr exposure) [47] which is similar to our findings in this study. Exposure of the HK-2 cells to the two MWCNTs in this study resulted in a dose dependant increase in both IL6 and IL8 following a 24 hr exposure. Our findings are similar to a recent study in which exposure of HEK 293 cells to two types of MWCNTs (80 and 150 nm) resulted in increased production of IL8 [48]. The HE oxidation assay showed no intracellular ROS following exposure to either of two MWCNTs. This is contradictory to findings from Reddy and colleagues in which cytotoxicity was associated with oxidative stress [48]. Finally we show that short term (4 hr) exposure of the HK-2 cells to the two carbon nanotubes at sub-lethal concentrations resulted in significant DNA damage. Barillet *et al* also witnessed small but significant genotoxicity following exposure of NRK-52E cells to 100 nm MWCNT which is similar to our findings in this study [43]. In another study exposure of lung A549 cells for 24 hr to -OH functionalized and pristine MWCNTs resulted in a concentration-dependent increase of direct DNA damage [49]. Similarly A549 cells exposed to MWCNTs for 24 hr resulted in an induction of direct DNA with statistical significance reached at 10 µg/ml [50].

**Minor revisions**

1. References have been modified.

2. We thank the author for this comment. This has been corrected.

3. Again the reviewer is correct. The error has been corrected.

4. All terms have been modified.
Reviewer 2 – Marcin Kruszewski

Major revisions

1. We have previously shown that the hepatocytes (which are not specialised phagocytic cells) engulf all of these NMs therefore the lack of difference in the uptake did not account for the subsequent differences in toxic impact to this cell type [23]. To our knowledge there is no published data that has investigated the uptake of any NMs by HK-2 cells. While it would be useful to address this question this was not a main aim of this manuscript.

2. We have modified the method section to stress that DHE is specific to the estimation of the production of superoxide ions. We agree with referee’s remark about the difficulty to discriminate the dead cells. However, we measured the cytotoxicity using WST-1 assay after 24 hours of treatment with NMs. The DHE analysis was performed after 4 hours of treatment to identify the earlier event underlying the cytotoxicity induced by the NMs. Observing cytograms of the cells treated with NMs compared to control. We do not see major alterations between populations, thus concluding that at this time point NMs are not inducing cellular death.

3. Numerous studies presented the FPG modified DNA damage using the method presented in this manuscript indicating that researchers accept this as a clear form of communicating the data (Gomes, et al., 2012; Kazimirova, et al., 2012; Kermanizadeh, et al., 2012; Sharma et al., 2012; Shukla, et al., 2013)**


The description of the concentrations used in this study has been clarified – the LC$_{20}$ ± one serial dilution has been used for the toxic NMs. For the NMs in which an LC$_{20}$ was not reached, the three top concentrations were utilised. All this information has been added to the method section and the legend for figure 3 (Please see below).

**Figure 3.** DNA damage expressed as percent of tail DNA following exposure of the HK-2 cells to the ENPRA panel of engineered nanomaterials. The cells were exposed to cell medium (control), 60 µM H$_2$O$_2$ and NMs for 4 hr. Values represent mean ± SEM (n=3), significance indicated by * = p<0.05 and ** = p<0.005, when material treatments are compared to the control. A) NM 300 B) NRCWE 002 C) NM 400 D) NRCWE 004 E) NM 402 F) NM 101 G) NM 110 H) NRCWE 003 I) NM 111 J) NRCWE 001. The LC$_{20}$ ± one serial dilution has been used for the majority of NMs (NM 110, NM 111, NM 300, NM 400, NM 402, NRCWE 003 and NRCWE 004). For NMs in which an LC$_{20}$ was not reached the three highest concentrations were utilised.

4. We thank the reviewer for the comment – The cytokine secretion following exposure to the highly toxic Ag NM has been expanded on in the discussion section. Please see below:

Furthermore we noted that there was a dose dependant increase in IL6 and IL8 levels following exposure of the cells to sub-lethal concentrations of the Ag NM, while higher concentrations were less effective due to cell death. Ag NM also induced a significant increase in genotoxicity following exposure to sub-lethal concentrations of the nanomaterial.

5. Zeta potential has been added to table 1

6. This has been modified in table 2

7. We have re-arranged as many graphs as possible to group similar materials into one graph (Figure 2). This was not possible for all data as the graphs become extremely busy and very difficult to interpret.
8. We have re-analysed all data and re-arranged all graphs according to their “biological effects”.

9. The discussion section has been significantly modified.

**Minor revisions**

1. The graphs have been modified.