Surface ligand dependent toxicity of zinc oxide nanoparticles in HepG2 cell model

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Abstract. Physicochemical properties of nanoparticles (NP) strongly affect their influence on cell behaviour, but can be significantly distorted by interactions with the proteins present in biological solutions. In this study we show how different surface functionalities of zinc oxide (ZnO) NP lead to changes in the size distribution and dissolution of the NP in serum containing cell culture media and how this impacts on NP toxicity. NPs capped with weakly bound large proteins undergo substantial transformations due to the exchange of the original surface ligands to the components of the cell culture media. Conversely, NP capped with a tight monolayer of small organic molecules or with covalently conjugated proteins show significantly higher stability. These differences in ligand exchange also affect the toxicity of the NP to the HepG2 liver cell model, with the NP capped with small organic molecules being more toxic than those capped with large proteins. This study highlights the importance of characterising NPs in biological media and the effect the media has during in-vitro analysis.

1. Introduction

To date there are over 1300 commercial products which incorporate NP, in a global market that is expected to be worth €30 billion by 2015¹. These products range from cosmetics and food additives through to paints or clothing and impact on almost all areas of human life. Inevitably this raises safety concerns due to the potential toxicity of the NP released from the products and absorbed, ingested or inhaled into the body². This can be a particular problem for organs such as the liver where bioaccumulation can rapidly occur following exposure to NP³, increasing and localising any toxic effect. Measurement of potential NP toxicity is usually performed using cell based screens. However, while this approach can be high throughput it is often challenging to study due to the interactions between the NP and serum proteins in the cell culture media⁴. These interactions lead to structural and functional changes of the NP, affecting their physicochemical properties and bioactivity. Therefore, it is important to understand these exchange reaction and how they affect the NP behaviour, cellular uptake and corresponding toxicity.

The ligand exchange reaction is directly related to the NP chemical composition, size, shape, net charge and most importantly, the initial surface functionality⁵. In this study we show how changes to the surface functionality of ZnO NP affect the physicochemical properties of the material over 24 hours, with size distribution profiles and ligand exchange reactions dependent upon the initial surface chemistry. We also show how different surface functionalities then impact the kinetics of cellular toxicity using impedance spectroscopy in a cellular model of the liver.
2. Experimental

2.1. Preparation of plain, APTMS, BSA and APTMS-BSA capped ZnO NP

Zinc oxide nanopowder (10-30nm, US Research Nanomaterials) was dispersed in water or capped with a selection of ligands using the following protocol. Weighted 5mg of powder was sonicated for 15min at room temperature (plain, APTMS and APTMS-BSA) or at 4°C (BSA) in an ultrasonic bath along with 50µl of isopropanol. Next, 5ml of ultrapure water (plain), 5ml of 2% 3-aminopropyltrimethoxysilane (APTMS) in isopropanol (APTMS and APTMS-BSA) or 5ml of 0.05% bovine serum albumin fraction V in ultrapure water (BSA) was introduced and the suspension was sonicated for 1h at room temperature (plain, APTMS and APTMS-BSA) or with a gradual temperature increase to room temperature (BSA). NP were then reacted for additional 24h at room temperature (plain and BSA) or at 60°C (APTMS and APTMS-BSA) with shaking, prior to purification from excess ligands and/or organic solvent residues by triple centrifugation/decantation (13,000rpm, 15min, room temperature). Capped NP were then redispersed by sonication in ultrapure water at 5mg/ml.

BSA was covalently conjugated to APTMS capped NP (APTMS-BSA) using EDC/sulfo-NHS coupling agents. Briefly, 0.34mg of APTMS capped NP was redispersed by sonication in 5ml of 0.01M borate buffer pH9. Then, 100µl of BSA fraction V stock (1mM in ultrapure water), 50µl of EDC (0.198M in ultrapure water) and 100µl of sulfo-NHS (0.198M in ultrapure water) were introduced. After, 48h stirring at room temperature, the conjugates were purified by triple centrifugation/decantation (13,000rpm, 15min, room temperature) and redispersed by sonication in ultrapure water at 5mg/ml.

2.2. ICP-MS analysis of the dissolution rate of plain and capped ZnO NP

The supernatants remaining after purification of plain, APTMS, BSA and APTMS-BSA ZnO NP (triple centrifugation/decantation at 13,000rpm, 15min, room temperature) were collected. NP were then stored for 24h at room temperature in the ultrapure water and centrifuged once more at 13,000rpm, 15min, room temperature to precipitate the NP. The supernatants containing dissolved ions were combined with the supernatants collected during the NP purification (also here the supernatants contained the dissolved ions only, since the NP were precipitated by centrifugation). The total content of $^{66}$Zn and $^{68}$Zn in the collected supernatants (diluted 1/50 by weight with HNO$_3$ just before analysis) were measured using an Agilent 7700x spectrometer operating in the He mode.

2.3. TEM imaging of plain and capped ZnO NP

10µl of NP suspension (5mg/ml) was deposited on a TEM grid (carbon film 400 mesh Cu 25, Agar Scientific) and air dried. TEM grids were imaged with Hitachi H7000 transmission electron microscope operating at 75kV voltage with 100,000x magnification (Biomedical Imaging Unit, Southampton, UK).

2.4. UV-visible and fluorescent spectroscopy of plain and capped ZnO NP

UV-visible and fluorescent spectra of ZnO NP suspensions in ultrapure water (0.1-1mg/ml) were recorder with Tecan Infinite M200 plate reader using high quality flat bottom black microplates (Sigma-Aldrich), over 350-400nm and 320-480nm range with 250nm excitation wavelength, respectively.

2.5. NTA sizing of plain and capped ZnO NP

Suspensions of NP (30-70µg/ml) were prepared in ultrapure water or cell culture media (EMEM 10% FBS) by sonication. The movement of NP in suspensions under Brownian motion was recorded with NanoSight NS500 over 60-90s at room temperature, then analyzed with NTA2.2 software using detection threshold value 9 and a minimum expected particle size of 30nm. The experiments were performed in triplicates with different batches of NP, processed with Microsoft Excel Office 2003 and are presented as mean ± standard deviation.
2.6. RT-CES testing of plain and capped ZnO NP toxicity in HepG2 cell model
HepG2 cells (LGC Standards), were seeded directly onto the gold microelectrode of the E-Plate (Acea Bioscience), at a density of 20,000 cells/well, as determined by an automated cell counter (ViCell, Beckman). The plate was then loaded into an impedance station housed at 37°C in 5% CO₂ environment and the impedance measurements were taken every hour over the duration (48h) of the experiment. Following the initial 24h after seeding the cells were exposed to the varied concentration of NP (30-70µg/ml) suspended in cell culture media (EMEM 10%FBS) and returned to the impedance station for the additional 24h. Untreated HepG2, as well as cells treated with APTMS (25µg/ml), BSA (50µg/ml) or 1% H₂O₂ served as controls. The experiments were performed in triplicates and are expressed as mean ± standard deviation.

3. Results and Discussion

3.1. Characterisation of ZnO NP suspensions in water.
ZnO NP with a primary crystal size of 10-30nm (as specified by the manufacturer) were functionalised with BSA, APTMS or a combination of both (APTMS-BSA). Using this approach, APTMS serves as a silinisation agent⁶, forming a tight monolayer of organics encapsulating the inorganic core and increasing protection against dissolution in aqueous media. Conversely, NP stabilized with loosely bound large protein (BSA) were less protected from ions and small organics in the surrounding environment and could readily undergo structural and functional changes, including destabilization and replacement of the original ligands⁵,⁷. Proteins, however, offered additional functionality, acting as targets that can be recognized by cells and in some cases modify their toxic response profiles⁸.

| Surface ligand   | Dissolution (%) | Size (nm)* | Shape   |
|------------------|-----------------|------------|---------|
| plain            | 6.35%           | 20-70      | near-spherical |
| APTMS            | 0.68%           | 18-60      | near-spherical |
| BSA              | 3.90%           | 16-65      | near-spherical |
| APTMS-BSA        | 2.95%           | 25-75      | near-spherical |

* as determined by TEM

To measure the effect of the capping process on the dissolution of zinc oxide, the NP samples were incubated at room temperature for 24 hours in ultrapure water and analysed with ICP-MS (Table1). In all cases the capping process provided protection to the NP against dissolution. Control NP with no functionalisation (plain) have a dissolution rate of 6.35% after 24 hours, compared to 0.68% after silinisation with APTMS and 3.9% following capping with BSA. This demonstrates the protective effect of the capping process. Surprisingly, the NP capped with a combination of APTMS and BSA (APTMS-BSA) had a higher dissolution rate (2.95%) than NP capped with APTMS alone. This is likely due to the BSA coupling step, which involved 48h incubation in a higher ionic strength environment with organics present which can destabilise ZnO NP and promoted dissolution⁹. Examination of NP using TEM (summary of TEM data displayed in the morphology section of Table1; images not shown) indicated that the capping process did not affect their morphology. All types of NP were near-spherical in shape with rough edges present and polydispersed in size with the diameter of primary crystals in a range between 16 and 75nm and no significant differences observed between the samples. It is important to note that the NP agglomerated/aggregated on the surface of the TEM grids regardless of the NP surface functionality, however the size of individual NP within the agglomerates/aggregates could still be measured manually (size data in Table1). The overall size of
agglomerates/aggregates is not given, since as it was not possible to distinguish between the agglomerates/aggregates formed in solution (if any) prior to deposition on the TEM grids and those formed during the grids preparation (Note: NP agglomerates/aggregates present in solution, as well as the primary NP and the relative proportion of the two, were measured with NTA, see ‘Ctrl’ in Figure 2).

Further characterisation of the capping process outcome was performed using fluorescence and UV-visible spectroscopy (Figure 1). These analysis showed that the fluorescent spectra of all types of ZnO NP had similar characteristics with a typical band around 400nm, meaning that the semiconductor properties were not lost or changed during capping\(^\text{10}\). Extinction characteristics of capped NP were altered only slightly in comparison to plain NP due to differences in the refractiveness of the surrounding media\(^\text{11}\). Clear sharpening of the UV-visible spectra and a slight blue shift of the extinction maxima indicate narrower size distribution of the overall population of NP (primary crystals and aggregates combined) and/or larger proportion of the primary fraction present.

![Fluorescence and UV-Visible Spectra](image)

**Figure 1.** Fluorescence (A) and UV-visible (B) spectra of plain, APTMS, BSA and APTMS-BSA ZnO NP suspensions in water.

### 3.2. Characterisation of ZnO NP suspensions in cell culture media.

Measurement of NP toxicity usually involves incubating the cells with the NP dispersed in serum containing cell culture media for a period of 24 hours. To measure how ZnO NP change during this time, capped and plain NP suspensions were examined for changes in the proportion of primary and aggregated fractions over 24 hours incubation at 37°C (Figure 2). Measurements of the area, height and distribution were performed using an image based nanoparticle tracking analysis (NTA) system to allow sizing of polydispersed samples (Figure 2). Dotted bars represent the characteristics of ZnO NP in water (Ctrl), solid bars show changes in NP size profiles during 24 hour incubation. In all cases the aggregated fraction accounts for a substantial proportion of the overall population of NP, with significantly lower number of plain compared to APTMS and BSA capped NP. When dispersed in the cell culture media APTMS and APTMS-BSA capped NP retained their original (Ctrl) size distribution profiles, while plain and BSA capped NP underwent substantial changes. At first the proportion of NP in the primary size fraction significantly dropped, which can be attributed to the initial stage of capping (plain) or ligand exchange (BSA) reactions with cell culture media components, happening directly on the NP surface and leading to destabilization and agglomeration/aggregation. After 4h in the culture media, the proportion of plain and BSA NP increased as the capping/ligand exchange propagated and the new organic corona was formed in equilibrium with the surrounding environment. Since no significant differences in the size distribution profiles of APTMS and APTMS-BSA NP over
time was observed, it can be assumed that silane protected NP are generally less prone to the ligand exchange reactions with the cell culture media components.

3.3. Characterisation of ZnO NP toxicity to liver cells.

The effect of capping on the toxicity of ZnO NP was measured in the HepG2 liver cell model using impedance spectroscopy (Figure 3), a label free method shown to be comparable to standard reagent based toxicity assays. Data for 48 hours is displayed, with the first 24 hours showing the growth of cells prior to exposure, indicated by an increase in impedance as the cells divide and increase in number. After 24 hours, the different capped NP suspensions in the cell culture media were introduced and elicit dose dependant toxic responses from the cells. Plain (uncapped) NP at 30µg/ml (lowest dose) did not have a direct toxic effect on the cells but changed their behaviour compared to control (untreated) samples, as indicated by a initial increase in impedance followed by a plateau through to the 48 hour time point. Plain NP at 40µg/ml were toxic to the cells and caused a rapid decrease in impedance over the first 8 hours of exposure as approximately 50% of the cells were killed by the NP. Concentrations of plain ZnO NP above 40µg/ml were extremely toxic killing the HepG2

![Figure 2](image-url) - Size changes of plain, APTMS, BSA and APTMS-BSA ZnO NP over 24h incubation in cell culture media, determined by the relative proportion of primary (0-100nm) and aggregated (>100nm) fractions. Area (top row) represents the sum of trapezoids under the size distribution curve, while height (middle) is the peak maximum. Position of the maximum peak determines the fraction diameter, while spread derived from the peak width (bottom). Ctrl are NP water suspensions.
cells within 6 hours. In comparison ZnO NP capped with either BSA or APTMS were more toxic than plain NP with even the lowest dose (30µg/ml) eliciting a mild toxic response to the cells and increased toxicity at 40µg/ml and above. Capping the ZnO NP with both APTMS and BSA reduced their toxicity with NP concentrations up to 50µg/ml having no toxicity, NP at a concentration of 60µg/ml having mild toxicity and the highest concentration (70µg/ml) having moderate toxicity. The BSA and APTMS ligands alone were not toxic to the cells, while Zn$^+$ ions have been shown previously to be non-toxic.

Changes in impedance provide valuable information about how the cells are responding to the presence of the NP. For all of the NP samples tested, there is an initial increase in impedance immediately after the NP have been added to the cells (after 24 hours). In previous reports this has been shown to occur due to biochemical changes and increased active transporter activity which modifies the cells impedance properties as they try to remove the internalised NP. These defence mechanisms are energy dependant and eventually exhaust the resources, so that after approximately 3 hours the toxic affect of the NP can be observed. At low concentrations of NP (30 and 40µg/ml) BSA and APTMS capped NP appear more toxic to cells than plain NP (cause bigger drop in the cell index value). This is most likely related to the higher content of NP in the primary size fraction within the first 4h, when the cell response takes place. In general, smaller (primary sized) NP are taken up by the cells more readily than larger aggregates and as more NP are internalised the effect on cells becomes stronger. When IC$_{50}$ values are taken into consideration (NP concentration at which cell impedance

![Figure 3](image-url)

Figure 3. Dynamic monitoring of changes in RT-CES impedance over 24h following exposure of sub-confluent HepG2 cells (after 24h of initial incubation) to 30-70 µg/ml suspensions of plain, APTMS, BSA and APTMS-BSA ZnO NP. The figure represents normalized cell index values with standard deviations less than 5% (not shown).
drops to 50% of the value recorded for untreated control), APTMS and BSA capped ZnO NP show the highest toxicity with IC$_{50}$ of 30µg/ml and 31µg/ml, respectively. Plain NP are slightly less toxic (IC$_{50}$ of 34 µg/ml) while APTMS-BSA are the least toxic to HepG2 cells, with an IC$_{50}$ of 58µg/ml. Significantly lower toxicity of APTMS-BSA NP compared to other types of NP, cannot be explained by the proportion of NP in the primary size range (higher than plain) or their diameter (not significantly different from other types of NP), thus it is most likely related to other characteristics of NP such as net charge and the effect of the functionality on the entry route of the NP into the cells and their overall uptake rate.

4. Conclusions

Changes in the surface functionality of NP achieved with different capping approaches affect the physicochemical behaviour of the NP in cell culture media and their cellular toxicity. Within the first 4h, which was the most important in generating the toxic response (as shown by impedance maximum peaks), relatively low proportion of BSA capped and plain ZnO NP was found in primary size fraction. This, combined with a high dissolution rate (ions which can be easily transported out of the cell) could be one reason why they showed slightly lower IC$_{50}$ values than APTMS capped NP. Significantly lower toxicity of APTMS-BSA NP than other types of NP, is most likely related to other than size characteristics of NP, e.g. differences in the net charge and the type of ligands present during the first 4h of the capping/exchange reaction in the cell culture media. Organic corona can have an effect on the NP entry route and the overall NP internalization rate, while appropriate surface functionality may even reduce the toxic responses.

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