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Nano-enhanced food contact materials and the in vitro toxicity to human intestinal cells of nano-ZnO at low dose

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Abstract: Nano Zinc Oxide (nZnO) has been shown to display antimicrobial effects which have led to its application in a number of areas such as antimicrobial surface coatings, anti bacterial wound dressings and more recently in polymer composite systems for use in food contact materials. Concerns have been raised due to the incorporation of nanoparticles in food packaging stemming from the possibility of repeated low dose direct exposure, through ingestion, primarily due to degradation and nanoparticle leaching from the polymer composite. To address these concerns, composites consisting of nZnO and polyethylene were formed using twin screw extrusion to mimic commercial methods of food contact material production. A leaching study was performed using Atomic Absorption Spectroscopy in order to determine the concentration of nZnO leached from the composite. Composite stability studies were performed and a leached nZnO concentration was evaluated. This concentration range was then utilised in a series of tests aimed at determining the toxicity response associated with nZnO when exposed to an intestinal model. In this study two human colorectal carcinoma cell lines, HT29 (ATCC No: HTB-38) and SW480 (ATCC No: CCL-228), were employed as a model to represent areas exposed by ingestion. These lines were exposed to a concentration range of nZnO which incorporated the concentration leached from the composites. The cytotoxic effects of nZnO were evaluated using four cytotoxic endpoints namely the Neutral Red, Alamar Blue, Coomassie Blue and MTT assays. The results of these studies are presented and their implications for the use on nano ZnO in direct food contact surfaces will be discussed.

1. Introduction

The rapid expansion of nano based technology into consumer goods represents a new dynamic between research and manufacturing with a move away from the traditionally slow incorporation of frontline research into commercial markets. According to the project on emerging nanotechnologies there are currently 806 identified 'nano' products, produced by 484 companies, located in 24 countries (www.nanotechproject.org). This 'nano' advance gives rise to impressive projections and statistics highlighting the importance of nanotechnology to national and international economies. However hidden behind the commercial and popular statistics is a significant lack of data on the toxicology of many of the commercially relevant nano-particles. Indeed the determination of the toxicity of a nanoparticle raises many questions ranging from physicochemical characterization protocols and

standards, through to the relevance of certain toxicological methods to specific particle[1]. Early nanotoxicology studies suffered difficulties in dose determination and in considering the effect of physical size and surface area [2,3], a significant flaw since these nano-properties can be directly related to many other properties such as reactivity, mobility and absorption which can influence the mechanism and the degree of toxicity [4]. To date, studies in the area have moved quickly to address these flaws however much of the reported literature has focused upon occupational exposure rather than risks facing consumers with the respiratory tract receiving considerable attention [5, 6]. While these studies are of great importance in promoting safe handling of nanomaterials, knowledge gaps still remain in the area of exposure to engineered nanomaterials by ingestion. To date only few studies explore the potential exposure to engineered nanoparticles by ingestion[7] and many of the current knowledge surrounding this type of exposure focus on very high dose exposures. Bing Wang et al [6] reported in 2006 an *in vivo* study of nano zinc oxide which was orally administered at high doses (5 g/kg body weight) to healthy adult mice. The results showed that nano zinc oxide gave rise to vomiting and lethargy in the mice with death occurring in two within one week which was deciphered to be due to intestinal obstruction. They also reported mice exposed to nano ZnO were found to have lesions in the heart and liver indicating the possible translocation in the body of the nanoparticles. It should be noted that these effects were the result of excessively high doses which are arguably not indicative of realistic exposure scenarios [8]. However the use of nanoparticles in consumer food packaging [9] and in some instances as an ingredient in food products has given rise to concerns about the possibility of repeated low dose direct exposure, through ingestion.

This study concentrates on nano Zinc Oxide, which is currently being utilised in novel food packaging materials as both a UV blocker and an anti-microbial agent. The anti-microbial activity of nZnO within the food contact material is said to hinder food spoilage by reducing the number of microbes in the food environment [9-12]. The ability of the nano material to leach from the food contact material and subsequently be ingested has yet to be addressed. In this study a composite of nZnO and Polyethylene was formed using twin screw extrusion in order to mimic one of the standard commercial methods of polymer based composite formation. Polyethylene was utilised due to its ubiquitous use in the production of consumer goods. From this a leaching study was performed on the material and the determined concentrations due to leaching were then used in a cytotoxic assessment. The low dose exposures directly mimic those that may be encountered during use of a nZnO activated food contact material.

The effects of low dose nZnO exposure to two colorectal cell lines were examined taking care to include the determined concentrations which occur by leaching. The cell lines used were SW480 and HT29 both of which originate from the lower intestine. These two lines were chosen due to their ease of culture and their reported use in other studies [13]. A number of assays namely Alamar Blue, Neutral Red, Coomassie Blue and MTT, were utilised to monitor any observed cellular response. Each assay measures a unique cellular function such as mitochondrial action, lysosomal action and general respiration therefore the use of a battery of assays allows for a greater understanding of the cytotoxic impact of nZnO on the two cell lines.

2. Methods

2.1. Characterisation of pristine materials

Nano particulate zinc oxide (ZnO) was purchased from Sigma Aldrich Ltd (Dublin, Ireland) and the particle size was characterised using three different methods namely Dynamic Light Scattering (Malvern nanoZS Zetasizer), Atomic Force Microscopy (Asylum Research Bio-AFM) and Transmission Electron Microscopy (Joel 100x). For DLS analysis nZnO was suspended in de-ionised water and measurements recorded at 10°C. For TEM analysis the nZnO was suspended in ethanol and drop cast on to Formvar copper grids (AGAR Scientific). Finally to perform AFM studies the nZnO was again dispersed in alcohol and for analysis samples were drop cast onto silicon wafers.

2.2. Composite formation

Composites were formed by hot melt extrusion. A Micro 27 Labscale Twin Screw Extruder (Leistritz Ltd) was used. A temperature of 150°C was set at the load-in point, the barrel containing the screws was set to 175°C and the temperature at the die was set to 180°C. The physical action of the screws along with the temperature in the barrel ensured a uniform dispersion of nZnO in polyethylene. The extrudate was cooled in water and then pelleted. Composites were produced using a range of nZnO loading fractions to produce materials of increasing percentage weight/weight ratios. The ratios were 0.25/0.5/1/2.5/5/10 % w/w.

2.3. Characterisation of Composites

The aforementioned composites were characterised using Atomic Force Microscopy and Fourier Transform Infrared Spectroscopy. For the AFM analysis the composites were heat pressed into thin disks which would provide a smooth surface for analysis. The FTIR analysis was also performed on the heat pressed composites.

2.4. Leaching Study

Leaching studies were performed by submersing 10g of the composite material in pellet form, in 20ml of 0.005M acetic acid which had a pH value of 4. This was done for each of the composites of 0.25-2.5% w/w nZnO. Acetic acid was used as it is a common additive in foods as well as an ingredient in some acidic foods (food additive code E260). These were stored in clean glass vials which were kept in normal light conditions at room temperature for 6 days. The concentration of the metal leached into the acid solution was measured using a Varian SpectrAA-200 Atomic Absorption Spectrometer. The samples were filtered through Whatmann number 5 filter paper in order to remove any large composite particles from solution which may interfere with the instrument. The instrument nebulises the analyte solution into an acetylene/air flame (with a flow rate of 2 dm³/minute and 13.5dm³/minute respectively) and any of the metal present is burned off and the absorption measured. The response from the instrument is measured against a standard curve attained by analysing solutions of known concentration.

2.5. Cytotoxicity assays.

For cytotoxicity evaluation cells were seeded in 96-well micro plates (Nunc, Denmark) in triplicate for each of the four time points studied 24, 48, 72, 96 hr. The plates were seeded at a density of 1.5 x 10⁵ cells/ml for 24hr, 5 x 10⁴ cells/ml for 48hr, 3 x 10⁴ cells/ml for 72hr and 2 x 10⁴ cells/ml for 96hr exposure. These densities were found to be optimal to achieve the desired confluence at the end of the exposure period. After an initial 24 hr of cell attachment, the media was removed and the plates were washed with 100 µl/well phosphate buffered saline (PBS). The cells were then treated with increasing concentrations of each nanomaterial and with a positive control of a 10% DMSO 90% media solution. These test solutions were prepared as follows: dispersions of the nano particles were created in fresh RPMI-F12 media supplemented with 10% foetal bovine serum (FBS) and 45 IU/ml penicillin and 45 g/ml streptomycin. An initial stock concentration of 1000 ppm was prepared by dispersing the nano ZnO using a sonic tip operating at 30 % amplitude in ten second burst followed by a ten second rest time to a total sonication time of 1 minute. Solutions of 500, 250, 100, 80, 40, 10, 0.1 ppm were then prepared from the 1000 ppm stock by serial dilution.

The cells were then incubated for the desired time period and the cytotoxic effects evaluated. For each independent experiment six replicate wells were used for control, six replicate wells were employed for the positive control and six replicate wells were used for each test concentration per micro plate. For cytotoxicity evaluation, fluorescence and absorbance were all quantified using a microplate reader (TECAN GENios, Grödig, Austria). Cytotoxicity was assessed using a number of assays as outlined below.

2.5.1. Alamar blue, neutral red, coomassie blue and MTT assays. The Alamar Blue, Neutral Red and Coomassie Blue assays were conducted subsequently on the same set of plates in order to measure metabolic activity, lysosomal activity and protein concentration according to the methods laid out by Casey et al [14, 15] A second series of plates were set up for the MTT assay. These plates were seeded and exposed identically to the first series of plates prepared for the Alamar Blue, Neutral Red, and Coomassie Blue assays and again the methods described by Casey et al used [14, 15].

2.6. Statistics

At least three independent experiments were conducted in triplicate for each cell line and toxicity endpoint. Test results for each assay were expressed as percentage of the unexposed control \pm standard deviation (SD). Control values were set as 100%. Differences between samples and the control were evaluated using the statistical analysis package SPSS 14.0. Statistically significant differences were set at $p \leq 0.05$. Normality of data was confirmed with Q-Q percentile plots and Kolmogorov-Smirnov tests. One-way analysis of variances [16] followed by Dunnett's multiple comparison tests were carried out for normally distributed samples with homogeneous variances.

3. Results

3.1. Characterisation

3.1.1. Pristine Materials. The nano Zinc Oxide particles were size characterised by three separate techniques DLS/AFM/TEM. Each one of these analyses showed that the average size of the particles fell well below the 100nm threshold to be considered on the nanoscale.

Table 1. Results of sizing of nano zinc oxide particles and the associated technique.

Method	Size (nm)
Dynamic Light Scattering	22 +/- 1.1
Transmission Electron Microscopy	42.7 +/- 9.9
Atomic Force Microscopy	51 +/- 9.1

3.1.2. Composite Characterisation. Atomic Force Microscopy was performed on the heat pressed composites. It was noted that the composites with a higher concentration of nano ZnO maintained a smoother topography. This could be due to the ZnO present altering the properties of the polymer matrix. A further investigation into this phenomenon is underway. IR spectroscopic analysis was performed on all the produced nano composites, the presence of the nZnO was seen to alter the spectroscopic band associated with a carbon carbon double bond in polymer (1740 Cm^{-1}), this perhaps giving an indication that the properties of the polymer are indeed altered by the presence of the nZnO. Further spectral and thermal studies will be performed to monitor any alterations in the polymer due to the presence of nZnO.

3.2. Leaching study

Composites were formed as described in section 2.2, the composite extrudate was noted to be an opaque white solid taking on a more brilliant white colour as the concentration of nZnO was increased. The leaching study consisted of analysing a series of standards on known concentration in order to produce a standard curve. From this the concentration of a number of unknowns could be calculated using the equation of the standard curve line. Composites of various mass loading fractions were analysed and a leached concentration of nZnO in the range of approximately 50 to 135ppm was determined, the amount of leached nZnO was also noted to be dependent on the loading fraction. In general a higher loading fraction resulted in a lower leached nZnO concentration. This perhaps giving an indication that the nZnO improved the chemical robustness of the composite but further studies are required to verify this theory.

3.3. Cytotoxicity Evaluation.

The following section will present the cytotoxicity data obtained from the colorimetric assays employed, namely Alamar Blue, Neutral Red, Coomassie Blue, MTT, to evaluate the effects of nano zinc oxide exposure to the two tested cell lines SW480 and HT29. Figure two gives an example of the type of cytotoxic response curves evaluated for nZnO as determined by the Alamar blue assay for the SW480 cell line.

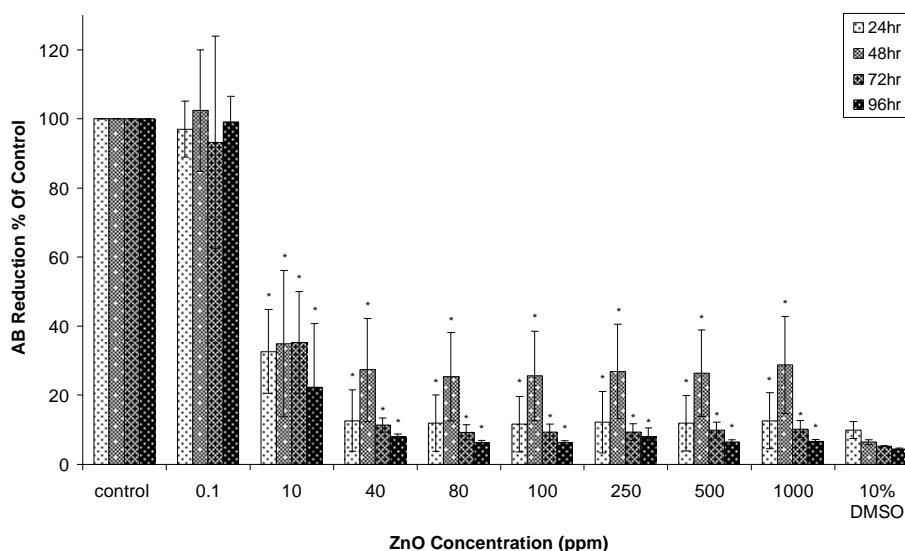


Figure 1. Cytotoxicity of nano ZnO to SW480 cells after 24, 48, 72 and 96 hour exposures determined by the Alamar Blue assay. Data are expressed as percent of control mean \pm SD of three independent experiments * denotes a statistically significant effect ($p \leq 0.05$).

Alamar Blue measures the innate metabolic activity of cultured cells and is routinely used in cytotoxicity screening, as can be seen in figure 1 there is an immediate cytotoxicological response in SW480 cells after exposure to nZnO. Cellular survival drops to approximately 30% after a 24 hour exposure to concentrations as low as 10 ppm, this survival rate drops further to approximately 10% at higher concentrations and longer exposure periods. Following the AB the neutral red assay was performed this assay monitors the Lysosomal activity of cultured cells. Again in this assay we see an immediate cytotoxicological effect at concentrations of 10 ppm and above (data not shown) resulting from a 24 hour exposure to nZnO with a maximum cellular survival rate of 20% being observed. Coomassie Blue is a broad spectrum protein stain which measures protein levels in cultured cells; it is detected by monitoring the absorbance of the Coomassie Blue after an exposure. In this assay remnant test particles increased the background absorbance so for this reason the highest test concentration cells were exposed to was that of 100 ppm nZnO. However in agreement with the previous AB and NR assays a cytotoxicity was noted at concentrations of 10 ppm and above after 24 hour exposure. The final assay performed was that of the MTT assay again this assays detection method is absorbance based so for this reason the highest tested nZnO concentration was 100 ppm. This assay measures the mitochondrial activity of cultured cells and is highly sensitive

The second cell line tested was that of the HT29 cell line overall the associated cytotoxicity resulting from nZnO exposure in this line was substantially lower than that of the SW480 cell line. Again the first assay performed was that of the AB assay (data not shown) in contrast to the results seen from the SW480 cells here cellular survival only reduced to 60% at the top tested concentration of 1000 ppm nZnO

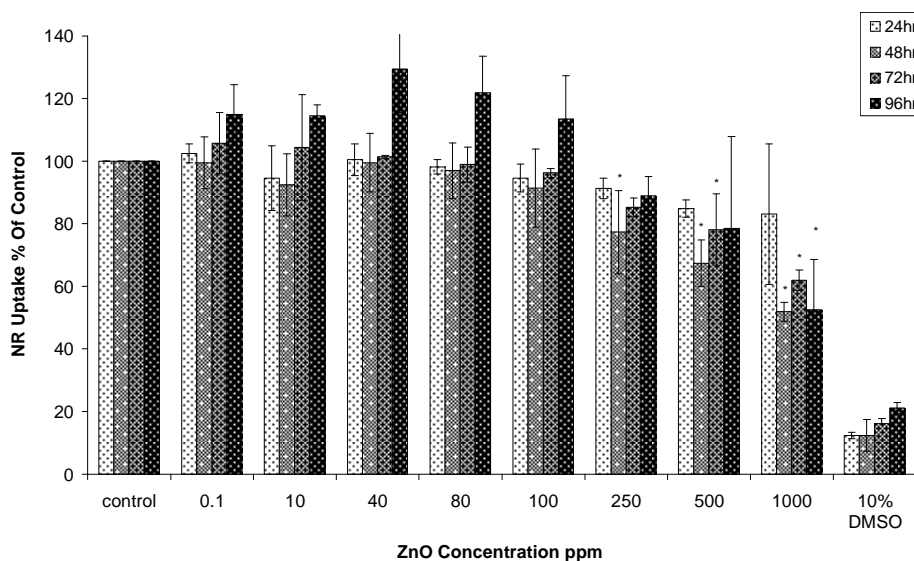


Figure 2. Cytotoxicity of nano ZnO to HT29 cells after 24, 48, 72 and 96 hour exposures determined by the NR assay. Data are expressed as percent of control mean \pm SD of three independent experiments, * denotes a statistically significant effect ($p \leq 0.05$).

Figure 2 displays the cytotoxic response resulting from nZnO exposure in the HT29 cell line as determined by the NR assay, again with this assay the HT29 appears to be more resilient to nZnO exposure. However cytotoxicity was observed at a lower concentration than that of the AB assay for this cell line, with statistically significant cytotoxicity being observed at concentrations of 250 ppm and above after 48 hour exposures. No data could be evaluated with this assay for the HT29 inconsistent results were continually acquired. The final assay performed for the HT29 cell line was that of the MTT (data not shown) as with the SW480 the highest test concentration studied with this assay was 100 ppm and no cytotoxicity was observed with this assay, which is in agreement with the AB and NR assays which indicated that a concentration above 250 ppm was required in the HT29 cell line to induce any cytotoxicity.

3.4. Light Microscopy.

A number of images were taken of both the SW480 and HT29 cells lines using a standard light microscope equipped with Nikon digital camera in order to assess any physical changes that may have occurred in the cells when exposed to nano ZnO. Furthermore these images were recorded to verify the experimental results of the cytotoxicity screening. For imaging a selection of the test concentrations (10 and 100 ppm) were prepared by sonication in cell culture media and exposed to each cell line for 24 hr. The cells were then washed with PBS and imaged. Figure 3 displays the light micrographs recorded figure 3 A and C display the unexposed control cells (HT29 and SW480). Upon exposure to the test particle clear differences became apparent; in both cases at the test concentration of 100 ppm (Figure 3 C and D) there were considerable morphological changes in both the tested cell lines with an almost complete loss of the cell monolayer. At the lower test concentration of 10 ppm differences between the responses of the two tested cell lines were noted with the test particles have a lesser effect on the HT29 cells in comparison to that of the SW480 which were significantly altered, this being in broad agreement with the results obtained from the cytotoxicity screening. Interestingly in the HT29 cell line higher amounts of cellular debris was noted in the exposures, this debris would be proteinaceous in nature and its presence may account for the inconsistent data acquired for the Comassie Blue assay in the HT29 cell line tests.

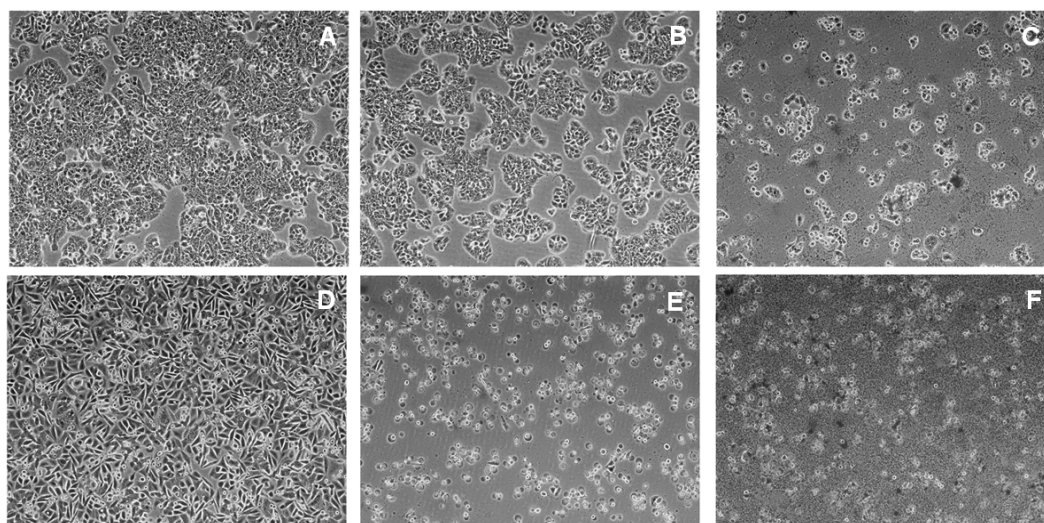


Figure 3. Light micrographs of (A) Unexposed HT29 cells (B) HT29 cells exposed to 10ppm ZnO for 24 hr (C) HT29 cells exposed to 100 ppm ZnO for 24 hr (D) Unexposed SW480 cells (E) SW480 cells exposed to 10 ppm ZnO for 24 hr (F) SW480 cells exposed to 100 ppm ZnO for 24 hr.

4. Discussion and conclusion

A series of nZnO/Polyethylene composites were formed. Polyethylene was chosen as due to its ubiquitous use in consumer goods. A leaching study was then performed by submersing a sample of the composite in a weak acid solution and analysing the leachate using AAS. Factoring in dilution the concentration of Zinc leached from the composite was seen to be between 50-135ppm for the low loading fraction composites. The higher loading fraction of 2.5% was seen to leach a concentration of approximately 50ppm. This lower leaching concentration may be due to the nZnO present altering the properties of the polymer matrix making it less susceptible to chemical stress however it should be noted that this leaching study was an initial investigation and supplementary experimentation is required in order to assess the leaching potential further. Cytotoxicity testing indicated a toxic response to the presence of nZnO in both HT29 and SW480 cell lines. The SW480 cell line was seen to be more susceptible to toxic effects. A significant response was noted in concentrations of 10ppm and above at exposure times as little as 24hrs. Both the MTT and Neutral Red assays confirm the low dose toxicity of the material to the cell line. It can be seen from the collected toxicological data the nZnO displays a cytotoxic effect to both the HT29 and SW480 cell lines at low concentrations, lower than those seen to leach from the composite materials. The concentration range of the leached Zinc is seen to be between 50-135 ppm which falls in the middle of the tested toxicity range.

Over the course of this study it has been clearly demonstrated that there is significant leaching of zinc from composite materials that mimic commercially available food contact materials and at the concentration ranges determined that nano particulate ZnO elicits a strong cytotoxic response. The Alamar blue and neutral red assays were noted to be the most sensitive of the colorimetric endpoints. These assays indicated that nano ZnO elicited a cytotoxic effect at concentrations below 10ppm in the SW480 line and above 40 ppm (Alamar Blue assay) in the HT29 cell line. These findings suggest further studies are needed to understand other underlying mechanisms by which nano ZnO elicits this cytotoxicity in intestinal cell lines. A recent study [17] has indicated that nano ZnO can cause significant genotoxic damage to human epithelial cells and work is ongoing to determine if a similar effect is occurring in the tested cell lines of this study. There is a possibility of the stability of the nano enhanced food contact material being compromised resulting in nanoparticle leaching which in turn may facilitate direct exposure to the intestinal tract by ingestion. The results of this study would lend themselves to the recommendation of a cautionary approach regarding the usage of nano ZnO in direct food contact materials.

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