CYTOTOXIC EFFECTS OF ZNO NANOPARTICLES INCORPORATED IN MESOPOROUS SILICA

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In vitro cytotoxic studies of nanoparticles use different cell line, incubation time and colorimetric assays. In this paper, we investigated the cytotoxicity of different concentrations of ZnO particles hosted in mesoporous silica in Human epidermoid cancer cells using the Trypan Blue exclusion test. The ZnO nanoparticles were prepared by incorporating different amount of zinc nitrate precursor into the channels of mesoporous silica SBA-15. Using Epics Beckman Coulter flowcytometer and FlowJo software the data were expressed as fractions of cells in the different cell cycle phases. The results show that all compounds have cytotoxic effects.

Keywords: ZnO, *in vitro*, cytotoxic, dye exclusion test, Trypan Blue

1. Introduction

The interactions of nanoparticles with microorganisms have recently attracted more attention and a wide range of antibacterial effects of zinc oxide nanoparticles have been reported [1,2]. As is clear from the literature, nanoparticles have unique biological response, selective toxicity and are generally regarded as a safe reagent to humans and animals.

Recent studies indicated that zinc oxide induces much greater cytotoxicity than non-metal nanoparticles and that the particle composition has a primary role in the cytotoxic effects [3,4].

ZnO nanoparticles have been reported to produce DNA damage on a human epidermal cell line (A431) and also induce cytotoxicity in the pulmonary epithelial cell line L2 [5,6].

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Ostrovsky *et al.* examined the cytotoxic effect of ZnO nanoparticles on various human cancer and normal cells and they found that the ZnO nanoparticles exerted a cytotoxic effect on the human glioma cell lines A172, U87, LNZ308, LN18 and LN229, whereas no cytotoxic effect was observed on normal human astrocytes. Similarly, the ZnO nanoparticles induced cell death in breast and prostate cancer cell lines while no major effect was observed in the respective normal breast and prostate cell lines [7].

The toxicity of the ZnO nanoparticles is dependent by size, dose [8], concentration and time [9]

In vitro cytotoxic studies of nanopaticles use different cell line, incubation time and colorimetric assays and because of this it is important to perform *in vitro* cytotoxic studies for each nanoparticles type [10,11].

There are three major categories of assays namely cytotoxic, genotoxic and alterations in gene expression assays which helps in evaluating the toxicity of nano particles in *in vitro* system [12]. Fadeel and Garcia-Bennett [13] reviewed the effectiveness and validity of assays for determining the toxicity and concluded that more than one assay may be required for nanotoxicity assessment. Monteiro-Riviere et al. [14] reported that the classical dye-based assays such as MTT assay produced invalid results with certain carbonaceous nanomaterials due to nanomaterial/ dye interactions. In addition the MTT assay failed to report toxicity of some porous silica microparticles due to spontaneous redox reactions where the MTT is reduced and nanoparticle surfaces are oxidized simultaneously [15]. Hence, it is concluded that assessment of nanoparticle toxicity should be carried out in case-to-case studies involving several accepted toxicity assays.

The Cytotoxic assays as Trypan Blue Exclusion Assay, *in vitro* cell viability assay, focuses on cell viability, plasma membrane integrity and cellular metabolism.

Cell cycle analysis is a method in cell biology that employs flow cytometry to distinguish cells in different phases of the cell cycle. When the cells pass through the flow cytometer's laser, a fluorescence pulse is generated that correlates with the amount of dye associated with the DNA and thus with the total amount of DNA in the cell [16].

The cell cycle consists of four distinct phases: G_1 phase, S phase (synthesis), G_2 phase (interphase) and M phase (mitosis). M phase is itself composed of two tightly coupled processes: mitosis, in which the cell's chromosomes are divided between the two sisters cells, and cytokinesis, in which the cell's cytoplasm divides in half forming distinct cells.

After cell division, each of the daughter cells begin the interphase of a new cycle. Although the various stages of interphase are not usually morphologically distinguishable, each phase of the cell cycle has a distinct set of specialized biochemical processes that prepare the cell for initiation of cell division.

Presence of disturbing agents like nanoparticles can alter the normal progression of cell cycle by DNA damages or intracellular homeostasis alteration leading to cell cycle arrest.

In this paper, we investigated the cytotoxicity of different concentrations of ZnO particles hosted in mesoporous silica in Hep-2 (Human epidermoid cancer cells) using the Trypan Blue Exclusion test.

As far as we know, the cytotoxicity of ZnO particles encapsulated in mesoporous silica SBA-15 has not been investigated until now.

As it was demonstrated, ZnO nanoparticles tend to aggregate due to the large surface area and high surface energy. In order to avoid this effect, we have incorporated the ZnO nanoparticles in a mesoporous silica SBA-15. The channels of nanoporous materials limit the growth of ZnO nanoparticles and diminish their agglomeration. These inorganic supports are chemically durable, can be easily handled, are non-toxic to human beings and are environmentally friendly [17]. Additionally, silica nanoparticles have already extensive applications in biomedical and biotechnological fields [18-21] due its toxic effect on endothelial cells[22].

The ZnO nanoparticles were prepared through nanocasting method by incorporating zinc nitrate precursor into the channels of mesoporous silica SBA-15. The synthesis of SBA-15 and the procedure of incorporating ZnO into the channels of SBA-15 were described in our previous work [23]. The samples used were denoted ZnO 1, ZnO 2 and ZnO 3 and have a different filling degree of the mesoporous silica channels with ZnO. (The filling degree increases in the following order: ZnO 1< ZnO 2 < ZnO 3).

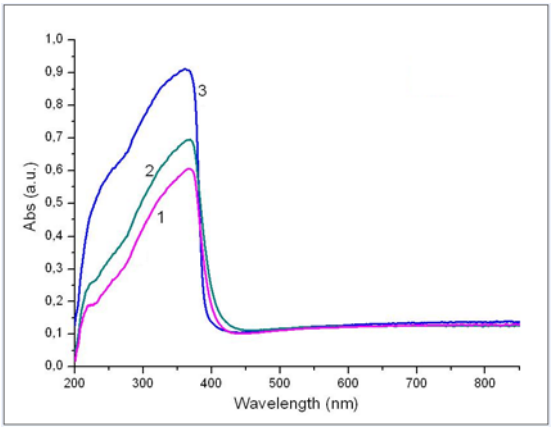


Fig.1 UV-Vis spectra of ZnO after repeated impregnation: 1: ZnO 1;2:ZnO 2;3:ZnO 3

The ZnO/SBA-15 nanocomposites shows a strong emission band at about 370 nm. As the ZnO loading increases, the intensity of this emission band increases.Usually, ZnO exhibits the UV near-band-edge emission peak at around

380 nm. Therefore, the emission band around 370 nm may be effect of the ZnO clusters encapsulated in the channels of SBA-15.

2. Experimental procedure

2.1. In Vitro Cell Viability/Cytotoxicity Studies

Cytotoxicity test was performed using Trypan Blue staining after the treatment with 100µg/mL and 1 mg/mL from each compound.

Trypan Blue it is a vital stain that is not absorbed by healthy viable cells. When cells are damaged or dead, Trypan Blue can enter the cell, causing strong absorption at ~605 nm wavelength , allowing dead cells to be counted. The method is sometimes referred to as the dye exclusion method. The dye exclusion test is based upon the concept that viable cells do not take up impermeable dyes (like Trypan Blue), but dead cells are permeable and take up the dye. Thus, the viable cells have a clear cytoplasm, whereas the cells coloured in blue are dead.

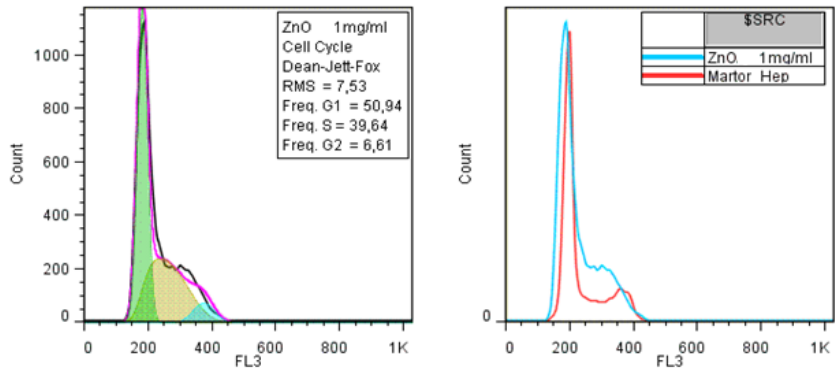
In brief, a freshly prepared solution of 50 µL Trypan Blue (0.05% in distilled water) was mixed to 50 µL of each cellular suspension during 5 min, spread onto a microscope slide and covered with a coverslip. Nonviable cells appear blue-stained. After 24 h, cell viability and cytotoxicity were monitored by counting viable (Trypan Blue excluding) cells in a hemacytometer.

2.2. Flow Cytometric Analysis of the Cell Cycle

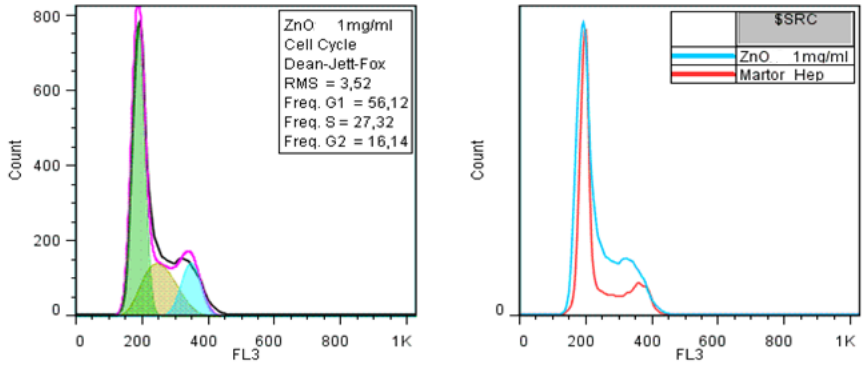
For cell cycle analysis, Hep-2 cell line (Human epidermoid cancer cells) was treated with 100µg/mL and 1 mg/ml from each compound and maintained for 24h at 37^0C, 5%CO_2 and humid condition. Thereafter, cells were harvested, washed in phosphate saline buffer (pH=7.5), fixed in 70% cold ethanol and maintained at -20^0C, over-night.

Each sample were washed in PBS, treated with 100µg/mL RNAse A for 15 minutes and coloured with 100µg/mL Trypan Blue solution as prepared by incubation at 37^0C, 1 hour. The incubation step is to ensure that the RNase has digested all the RNA, which otherwise would interfere with the DNA signal. After staining of cells with Trypan Blue the acquisition was done using Epics Beckman Coulter flowcytometer. Data were analyzed using FlowJo software and expressed as fractions of cells in the different cell cycle phases.

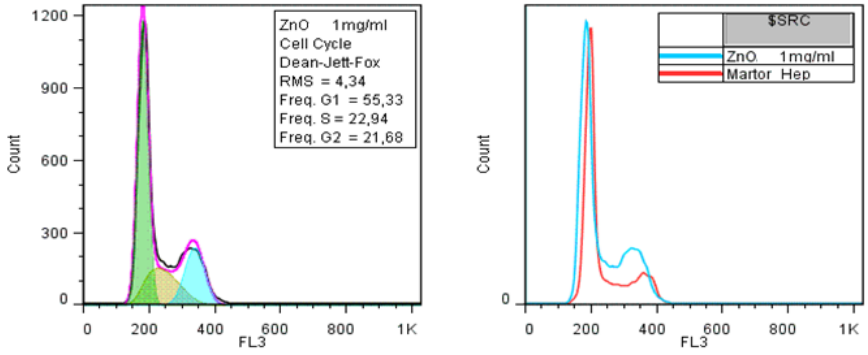
Histograms from Fig.2 represent distributions among cells in the population fluorescence intensity after staining with Trypan Blue. The ordinate gives the number of cells per channel, and the abscissa gives the relative fluorescence intensity, which is proportional to DNA content. In this histograms, the large peak contains cells with G1 DNA content and the smaller peaks represents cells with the DNA content of cells in the late S, G2, and M part of the cell cycle.



a) ZnO 1



b) ZnO 2



c) ZnO 3

Fig.2. Histograms representing distributions among cells in the population fluorescence intensity after staining with Trypan Blue: a)- ZnO 1;b)-ZnO 2 and c)-ZnO 3

From these histograms we can see that, even if the percentage of cells in the S phase decreases irregular, it remains still bigger than that of control. The

increased percentage of cells in the S phase when treated with ZnO nanoparticles indicates that the cells with DNA damaged were arrested in the checkpoint G2/M.

3. Results and discussions

We investigated the effect of ZnO nanoparticles encapsulated in mesoporous silica on the cell cycle distribution to evaluate their toxicity.

The cell cycle consists of interphases (G1, S, and G2) and mitosis (M). During the G1 period, cells increase in size, produce RNA, and synthesize proteins for DNA formation. In normal conditions, during the S phase, DNA replication occurs and the cells continue to grow, producing new proteins at the G2 phase. Nuclear and cytoplasmic division occurs at the M stage. The presence of ZnO nanoparticles alters the normal cellular cycle by damaging DNA and cellular cycle arrest.

From our study we can conclude that the cell viability/cytotoxicity of Hep-2 cells treated with ZnO nanoparticles encapsulated in mesoporous silica SBA-15 in different concentration depends on the concentration of the nanoparticles (Fig. 3) and the time of exposure (Fig. 4).

As indicated in Fig. 3, when the concentration of ZnO nanoparticles was 100 µg/mL, cell viability was decreased to 87,01% for ZnO1, 89,61% for ZnO2 and 86,84% for ZnO3. When the concentration of ZnO nanoparticles was increased to 1mg/mL the cell viability decreased to 76,19%, 76,32 % si 83,33% for ZnO1, ZnO2 and ZnO3 respectively, values significantly lower than that of control.

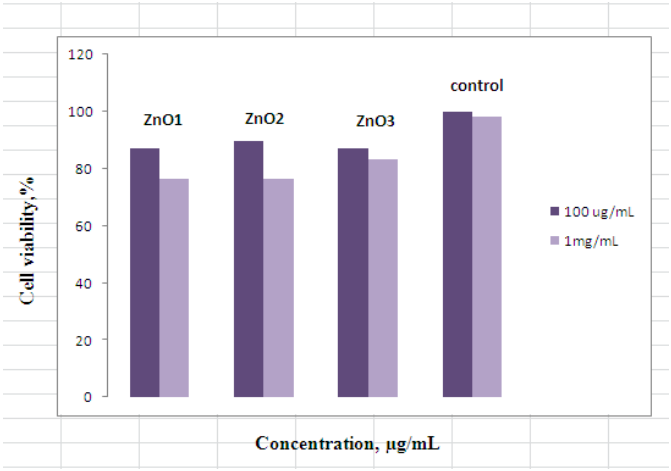


Fig.3. Cell viability/cytotoxicity of Hep-2 cells depending on the concentration of the ZnO nanoparticles

Fig. 4 shows the cell viability treated with different concentrations of ZnO nanoparticles depending on the exposure time. As it can see, cell viability decreases as the exposure time is longer.

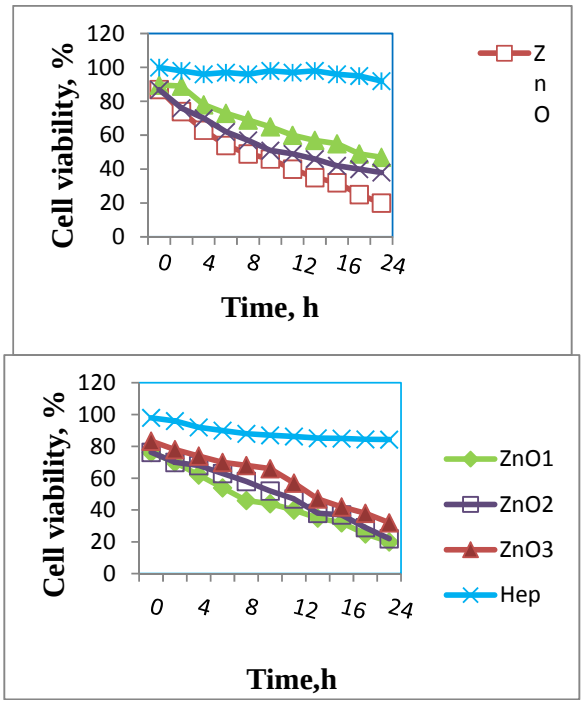


Fig.4. Cell viability/cytotoxicity of Hep-2 cells treated with 100 µg/mL(left) and 1mg/mL (right) ZnO nanoparticles depending on the exposure time

From Table 1 we can see that, the percentage of cells in G2/M phase increased progressively depending on the concentration of nanoparticles, while in G0/G1 and S phase the percentage of cells declined irregular. The increased percentage of cells in the G2/M phase when treated with ZnO nanoparticles indicates the inhibition of DNA synthesis and cell cycle arrest.

Table 1

The percent of *viable* cell estimated by trypan blue dye exclusion test, after 24h treatment with compounds ZnO1, ZnO2 and ZnO3

	G0/G1	S	G2/M
Hep (martor)	64.37	20.84	13.31
ZnO 1	50.94	39.64	6.61
ZnO 2	56.12	27.32	16.14
ZnO 3	55.33	22.94	21.68

Previous study confirmed that nanoparticles can induce generation of reactive oxygen species which lead directly to DNA damage and cell cycle arrest [24]. The cellular response to DNA damage, commonly known as DDR, encompasses multiple repair mechanisms and checkpoint responses that can delay cell cycle progressing or modulate DNA replication [25].

It had been reported that silica nanoparticles could induce DDR, mutagenic effects and cell cycle arrest in various cell lines [26 - 28]. In response to DNA damage, cells launch cell cycle checkpoints to detect and repair damaged DNA to maintain the genome stability [29].

When cells have DNA damage to be repaired or DNA replication is not complete, these checkpoints will arrest cell cycle at one of the G0/G1, S or G2/M phase. The G2/M phase has played an important role in mitotic processes. G2/M DNA damage checkpoint serves to prevent the cell from entering mitosis (M phase) with genomic DNA damage [30]. This kind of cell cycle delay could offer more time for the repair of DNA damage and avoid gene mutation [31]. However, when the DNA injuries of cells were so severe that exceed the cellular repair capacity, apoptosis would occur. Cell cycle checkpoints are pivotal mechanisms safeguarding genome stability. Cells that contain defects in the checkpoints are predisposed to genome instability and neoplastic transformation [32].

The potential mechanisms that explain why ZnO NPs exert toxic effects would be: based on oxidative stress, coordination effects and non-homeostasis effects [33].

Xia et al. reported that ZnO nanoparticles induce generation of reactive oxygen species which can lead to cell death when the antioxidative capacity of the cell is exceeded.

In the case of ZnO nanoparticles, generation of reactive oxygen species has been attributed to their semiconductor and nanolevel characteristics, which lead to generation of reactive oxygen species even in the absence of light [34].

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA.

Coordination effects due to interactions between metal oxide nanoparticles and proteins can directly and indirectly promote cellular DNA damage. ZnO nanoparticles can induce physical damage to genetic material because, if they are small enough, they can diffuse through the nuclear pore complexes or gain access when the nuclear membrane dissolves during mitosis [35].

Zn^{2+} is a vital component of enzymes and proteins and play significant roles in maintaining organism's homeostasis and those low or high levels of Zn can disrupt homeostatic mechanisms. In addition, ZnO nanoparticles may release

Zn^{2+} that increases the local concentrations of metal ions and can disrupt metal cation cellular homeostasis.

On the other hand, it is believed that ZnO nanoparticles have bactericidal properties primarily due to its photocatalytic activity. It was observed that ZnO shows bactericidal properties also in case of complete absence of light [36]. ZnO can absorb light (UV or visible) which induces a separation of charge, generating a hole ($h+$) in the valence band and an electron ($e–$) in the conduction band:

$$ZnO + h\upsilon \rightarrow e^- + h^+ \text{ (on the surface of ZnO particles)}$$

At the surface of the excited ZnO particle, the valence band holes abstract electrons from water and/or hydroxyl ions, generating hydroxyl radicals (OH•). In addition, electrons can reduce O_2 to produce the superoxide anion $O_2^{-\bullet}$:

$$h^+ + H_2O \rightarrow .\ OH\bullet + H^+$$

$$e^- + O_2 \rightarrow O_2^{-\bullet}$$

The obtained OH• and $O_2^{-\bullet}$ can induce lipid peroxidation in membranes, DNA damage due to strand breakage or oxidized nucleotides and oxidation of amino acids and protein catalytic centers [37]:

$$(O_2^{-\bullet}, OH\bullet) + \text{organic material} \rightarrow CO_2 + H_2O$$

4. Conclusions

The cytotoxicity of different concentrations of ZnO particles hosted in mesoporous silica in Human epidermoid cancer cells was investigated using Trypan Blue exclusion test. The histograms show that engineered nanoparticles have high toxicity which is in agreement with previous studies by other research groups.

Moreover, our data indicated that the ZnO nanoparticles inhibited Hep-2 cells proliferation by inducing G2/M arrest (Fig. 2 and Table 1). This could be beneficial in the use of these nanoparticles as an antibacterial agent in various industrial applications: replacement of some antibiotics or even as additive in film forming materials.

In the future, we plan to conduct more studies to understand the interaction of ZnO nanoparticles and protein and correlate that to the degree of toxicity.

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