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# In Vitro Cytotoxicity of $Gd_2O_3$ Nanoparticles with Diethylene Glycol Polymer in human melanoma Cell Line.

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**ABSTRACT**

Nanoparticles are exceptional in that their physical and chemical properties enable many promising medicinal applications. It is widely accepted that nanoparticles should be thoroughly tested for health hazards or nanotoxicity. The purpose of this study was to assess in vitro cytotoxicity of Gadolinium oxide nanoparticles in Human melanoma Cell Line. The effects of magnetic nanoparticles on this cell were evaluated by light microscopy and by standard cytotoxicity assays. Our results demonstrate a concentration-dependent toxicity for all types of particles tested. Our results suggest that this cell lines provides valuable models to assess the cytotoxicity of nanoparticles in human melanoma Cell Line in vitro.

**Keywords:** Nanoparticles; Cytotoxicity; Cell line; Gadolinium oxide; Diethylene glycol.

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## INTRODUCTION

Nanotechnology involves the design and management of materials at the nanoscale level to create distinctive products that develop novel properties. [1] Bionanomaterials, which are by definition in the 1–100 nm range, have been used to create materials that have novel physical/chemical properties and functions based on their advantageous, miniscule size. [2,3] Nanoparticles have been planned for the treatment and diagnosis of many diseases that need constant drug concentration in the blood or drug targeting to specific cells or organs. [4,5]

The development of suitable magnetic resonance molecular imaging (MRMI) contrast agents is, therefore, a very important and absorbing field of research. If formulated properly with other materials, nanomaterials may provide greater stability and efficiency for propellant systems.[6] Despite the wide application of nanomaterials, there is a serious lack of information concerning the impact of manufactured nanomaterials on human health. [7] Typically, after systemic administration, the nanoparticles are small enough to penetrate even very small capillaries throughout the body, and therefore they offer the most effective approach to distribution in certain tissues. Because nano- particles can pass through biological membranes, they can affect the physiology of any cell in an animal body. [8,9] This study would be involved with nanoparticles composed gadolinium (III) oxide ($Gd_2O_3$) with diethylene glycol polymer. $Gd_2O_3$ nanoparticles with Diethylene Glycol Polymer could produce a good MR signal and therefore could be a useful potential contrast medium for cell tracking in magnetic resonance molecular imaging(MRMI). [10] In this study, $Gd_2O_3$ nanoparticles were used as a model nanomaterial for the evaluation of invitro potential toxicity. The purpose of this study was to assess in vitro cytotoxicity of $Gd_2O_3$ nanoparticles in Human melanoma Cell Line as models to assess nanotoxicity invitro. The effects of nanoparticle on this cell was evaluated using light microscopy and by standard cytotoxicity assays.

## EXPERIMENTAL

### Synthesis of $Gd_2O_3$ Nanoparticles

Gadolinium oxide nanoparticles were synthesized by the polyol method started from $Gd_2O_3$ reacting with HCl and resulting to GdCl36H2O ( 2.5 mmol) was dissolved in 12.5 ml DEG (diethylene glycol) by heating the mixture to 140 C. Solid NaOH (3.125mmol) was dissolved in 12.5 ml DEG and subsequently added to the Gd containing solution. The temperature of the mixture was raised to 180 C and held constant for 4 h under reflux and magnetic stirring yielding a colloid. The concentrations for samples were 0.1, 0.3, 0.6, 0.9, 1.2 ,1.5, 1.8 and 2.5 mM and the volume for each sample was 5 ml. The size and morphological structure of this Nano particle determined by particle size analysis device(zeta sizer) and Transmission Electronic Microscope(TEM).

### Cell Line

The SK-MEL-3(Human skin, malignant melanoma, cell line obtained from National Cell Bank of Iran, Pasteure Institute of Iran were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 0.03% L-Glutamine and 1% penicillin/streptomycin (Gibco-BRL, MD, USA) at 37°C in a 5% $CO_2$ atmosphere.

### Microculture tetrazolium test (MTT assay)

The inhibitory effect of $Gd_2O_3$ on growth and proliferation of SKMEL-3 cell line was assessed at uptake of thiazolyl blue tetrazolium bromide (MTT, Sigma) by viable cells. [11] Cells were plated on to 96-well plates (Orange Scientific, Brussels, Belgium) at a density of 1.5 x $10^4$ cell/100µl/well. After incubation at 37°C for 24 h, the medium was replaced with either control medium or medium containing specialized concentration $Gd_2O_3$ for 2, 8 and 24 h. The concentration of $Gd_2O_3$ for cell culture treatment was 0.1, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.5 mM respectively and the concentration of zero means negative control. 50 µl of MTT solution (0.5 mg/ml) was added to each well and then cells incubated at 37°C for 3 h. Following solubilization of precipitated formazan with 100 µl DMSO, the optical densitometry was measured at a wavelength of 570 nm. The inhibition rate (IR) was evaluated using the following equation: IR (%) = 1- $OD_{exp}/OD_{con}$ ×100, where $OD_{exp}$ and $OD_{con}$ are the optical densitometries of treated and untreated cells, respectively. The viability rate of $Gd_2O_3$ was evaluated using the following equation: Viability (%) = 100 - IR (%). [12]

**LDH leakage**

LDH assay was performed for detection of cytotoxicity index of $Gd_2O_3$-DEG in the cell medium by using a commercially available kit (Roche Applied Science).

Serum Lactate Dehydrogenase (LDH) Assay-Serum stored at 20 °C was used for this assay, [2] which was performed according to the manufacturer's protocol (Roche Applied Science). Briefly, after the exposure of SKMEL-3 cell line to and $Gd_2O_3$-DEG for 2 h, 100 µl of supernantant was transferred into an optically clear 96-well flat-bottomed microtiter plate. The concentration of $Gd_2O_3$ for cell culture treatment was 0.1, 0.3, 0.9, 1.5, 2.5 mM respectively and the concentration of zero means negative control. To determine LDH activity, 100 µl of reaction mixture was added to each well and incubated for 30 min at 15–25 °C. Control group is %0.1 DMSO (Dimethyl sulfoxide). The spectrophotometer was calibrated to zero absorbance using culture medium without cells. The relative LDH leakage (%) related to control wells containing cell culture medium without nanoparticles or PBS as a vehicle was calculated by $[A]_{test}/[A]_{control}\times100$.

Where $[A]_{test}$ is the absorbance of the test sample and $[A]_{control}$ is the absorbance of the control sample. The absorbance of the samples was measured at 490 nm using an ELISA reader. Released LDH in culture supernatants results in the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of color formed is proportional to the number of lysed cells. Cytotoxicity is expressed relative to the basal LDH release by untreated control cells.

In addition, cell viability after incubation with $Gd_2O_3$–DEG was studied with trypan blue. Incubation concentrations used were 0.5 and 2.5 mM and cells were dyed with trypan blue and counted in a Burker chamber. One set of samples was washed after 2 h of incubation and one set of samples was kept unwashed. The cell samples were then kept in culture for 8 days and viability was monitored directly after 2 h of incubation and after 24h. [13]

**Atomic absorption spectroscopy (AAS) assay**

At the end of Gd NP exposure, cells were washed twice in PBS, trypsinized, spun down and resuspended in nitric acid (69%). Cells were lysed at room temperature by sonication for 15 min in a water bath and allowed to digest for 1 h. Finally, samples were centrifuged at 5,000×*g* for 2 min to eliminate debris. Supernatants were diluted to a final concentration of 5% HNO and AAS was performed as described above.

**Side scatter measurements**

After Gd NP exposure, cells were trypsinized, centrifuged and resuspended in PBS. The light side scatter intensity was measured by flowcytometry (Quanta SC MPL, Beckman Coulter). Cellular debris was gated out, and the mean side scatter was recorded by the Quanta SC MPL Analysis software based on 20,000 events.

**Statistical analysis**

All data were presented as mean ± standard deviation (SD). Student's t-test for means comparison of two samples was carried out. Statistical significance was ascertained when p value was less than 0.05.

## RESULTS

Characterisation Test: For particle size determination; particle size analysis device(zeta sizer) showed $Gd_2O_3$nanoparticles to be 30 nm, Fig.1(A), Meanwhile, the size and morphological structure of the nanoparticle also determined by Transmission Electronic Microscope(TEM)(A902 TEM CEM model), Fig.1(B).

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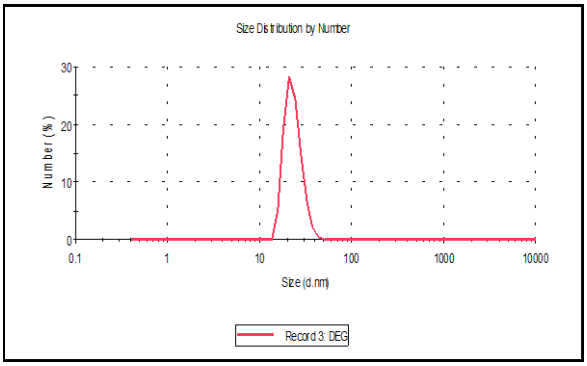
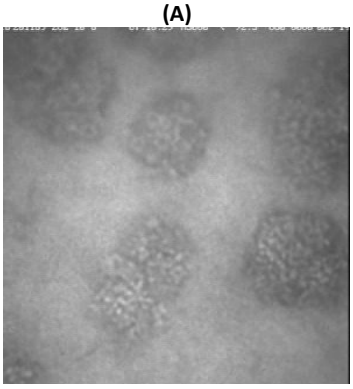



(A)



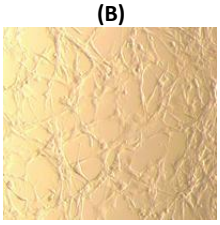
(B)

**Figure 1(A): Size measurement of the $Gd_2O_3$-DEG and (B): TEM photomicrograph of a single gadolinium nanoparticle.**

**Cell Morphology**

The general morphology of the cell incubated with nanoparticles in phase-contrast microscopy is shown in Figure 2.

(A) (B) (C)



(D)

**Figure 2: Morphology of the cells (A) SK-MEL-3control cell (B) SK-MEL-3 cells after incubation with $Gd_2O_3$DEG for 2 h (C) SK-MEL-3 cells after incubation with $Gd_2O_3$-DEG for 8 h (D) SK-MEL-3 cells after incubation with $Gd_2O_3$-DEG for 24h.**

The fig2.B,C,D show that the cells were well spread, and there was no distinct change in morphology after 2, 8, 24 h of incubation with $Gd_2O_3$-DEG nanoparticles relative.

**Gd NP uptake**

It has been proposed that the intracellular concentration of NPs is reflected in the intensity of light side scatter measured by flow cytometry. [14] Using this experimental approach, a dose dependent increase in

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cellular uptake of Gd NPs was detected at doses from 5 to 10µg/ml after 24-h NP exposure (Fig. 3a). A similar dose-dependent increase in side scatter was detected when the cells had been pretreated by NAC for 1 h, suggesting that the increased cellular granularity is not an artifact caused by dying cells.To further verify the uptake of Gd in SKMEL-3cells at different time points and doses, AAS analyses were employed (Fig. 3b). Gd could not be detected in controls and cells exposed to Gd NP for 1 min. However, at 4 and 24 h, a dose- and time-dependent accumulation of Gd could be measured in SKMEL-3 cell lysates.

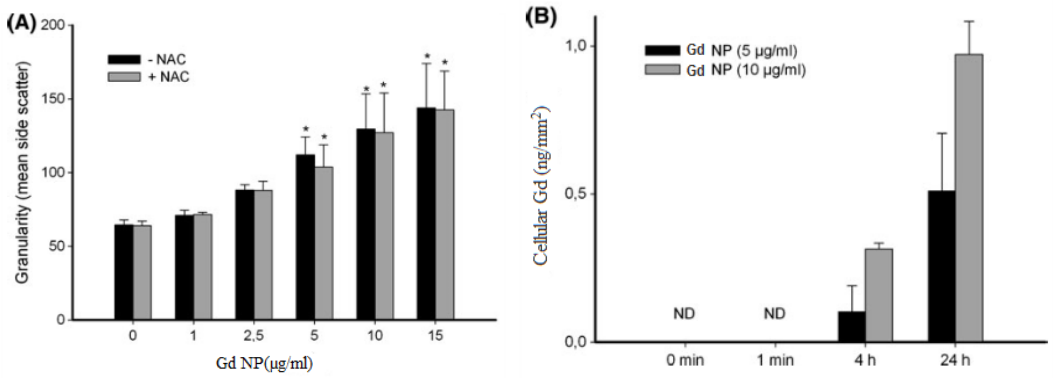
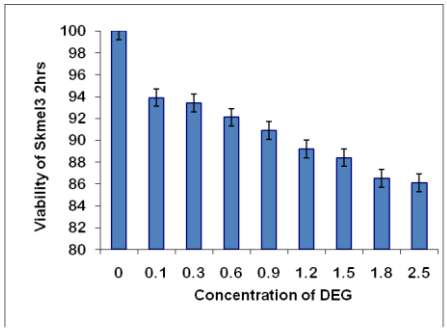


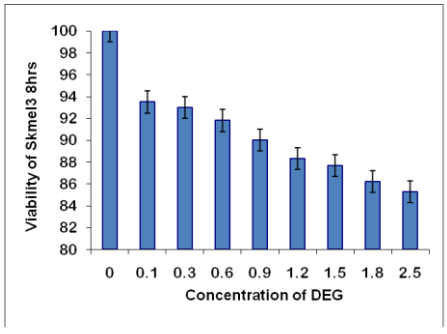

**Figure 3: Gd NP uptake (A) Cells were pretreated for 1 h in media ± NAC (10 mM) and exposed to different concentrations of Gd NPs for 24 h. After exposure, the cells were assayed for light side scatter intensity by flowcytometry (B) Cells were treated with Gd NPs at different time points, and the Gd concentration of cell lysates was determined by AAS. The data are expressed as mean ±SD of three independent experiments. *Asterisks* denote significant (*p* <0.05) difference from the untreated control. ND = not detectable.**

**Microculture tetrazolium test (MTT assay)**

The mitochondrial function of the cells was measured by means of the MTT assay after culturing in presence of the nanoparticles for 2, 8 and 24 h. As is evident from Figure 4, the gadolinium(III) oxide($Gd_2O_3$) nanoparticles with diethylene glycol polymer (DEG) had no significant effect.




**(A)**




**(B)**

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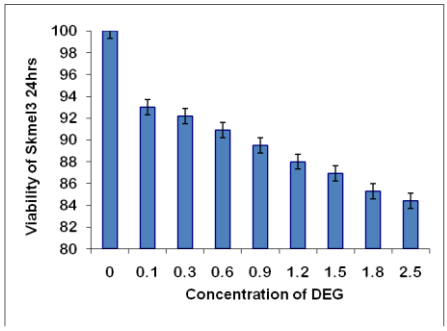


**(C)**

**Figure 4: viability of SK-MEL-3cells (A) after 2hr incubation with $Gd_2O_3$-DEG (B) after 8hr incubation with $Gd_2O_3$-DEG (C) after 24hr incubation with $Gd_2O_3$-DEG .**

**LDH leakage**

Fig5 shows that soluble $Gd_2O_3$-DEG have no effect on the plasma membrane at any of the concentrations tested.

Viability with trypan blue observations showed that incubation with $Gd_2O_3$-DEG for 2 h did not affect cell line. Neither washed samples nor samples where $Gd_2O_3$was left in the culture were affected in any significant way.

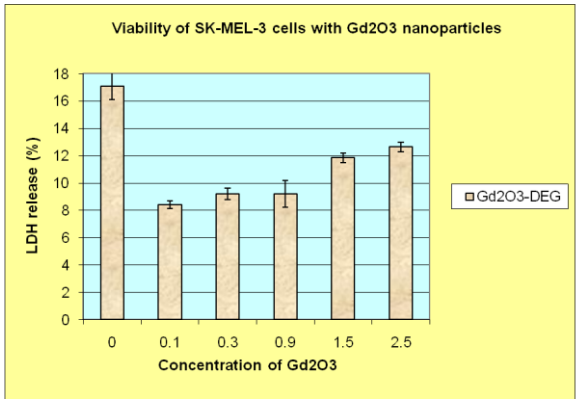


**Figure 5: LDH membrane leakage in presence of different concentrations of $Gd_2O_3$ nanoparticle.**

**DISCUSSION**

Magnetic resonance imaging (MRI) is an really important imaging modality because it does not employ ionising radiation, has excellent spatial resolution and can be used to image wide areas of interest. Its usefulness can be enhanced further by using contrast agents with magnetic susceptibility. Gadolinium (Gd) is currently the most common MRI contrast agent. For MRI to compete with, and to be superior, molecular imaging techniques such as positron emission tomography (PET) and single photon emission computed tomography (SPECT), special contrast agents consisting of nanometer-scale particles carrying high delivering of gadolinium will be needed. The development of suitable magnetic resonance molecular imaging (MRMI) contrast agents is, therefore, a very important and absorbing field of research. [15,16]

Gadolinium (Gd) is a well known paramagnetic contrast agent the natural toxicity of which is conventionally counteracted by using it in a chelated form such as diethylenetriamine pentaacetic acid (Gd-DTPA) or tetraazacyclododecanetetraacetic acid ( Gd-DOTA). [17]

Magnetic nanoparticle probes are emerging as a class of novel contrast and tracking agents for medical imaging. When used as a contrast agent for magnetic resonance imaging (MRI), allow researchers and

clinicians to enhance the tissue contrast of an area of interest by increasing the relaxation rate of water. [18-23].

Development of an ideal paramagnetic nanoparticle-based contrast agent is a 'hot topic' in current research focused on making MRMI a clinically feasible and superior form of diagnostic molecular imaging. Paramagnetic nanoparticles such as gadolinium have the potential to greatly enhance the sensitivity, and therefore clinical usefulness, of MRI by enabling imaging at cellular and subcellular levels. [24-29, 10] This study would be involved with nanoparticles composed gadolinium (III) oxide ($Gd_2O_3$) with diethylene glycol polymer.

In this study, we used SKMEL-3 cell line to evaluate the toxicity of $Gd_2O_3$nanoparticles. However, TEM is a time consuming qualitative method for determining NP uptake. Accordingly, we used the light side scatter (granularity)of NP-exposed cells to demonstrate Ag NP uptake [14] which was verified by AAS measurements on cell lysates.

We used parameters widely used in toxicological studies, such as the ability of mitochondria to reduce MTT, the integrity of the plasma membrane. [30, 31]

The proliferation/viability of $Gd_2O_3$ nanoparticles was measured by MTT assay after culturing for 2, 8 and 24 hours. As it is evident from fig.2, After 24 hours, cell line was found to be more than 100% viable relative to control cells at $Gd_2O_3$-DEG nanoparticles. Toxicity of $Gd_2O_3$-DEG nanoparticles was sufficiently low since no significant decrease in cell viability was observed in cells interacting with nanoparticles for prolonged periods.

## CONCLUSIONS

The results from the MTT assay and LDH assay suggest that the $Gd_2O_3$-DEG nanoparticles are non-toxic to cells and do not cause any apparent harm to cells. Our results suggest that this cell lines provides valuable models to assess the cytotoxicity of nanoparticles in vitro.

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