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# CYTOTOXICITY OF MAGNETIC NANOPARTICLES ON NORMAL AND MALIGNANT HUMAN SKIN CELLS

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Magnetic nanoparticles have received considerable attention in nanomedicine due to their potential application as therapeutic or diagnostic tools based on their particular properties. However, prior to clinical application investigating the effect of these nanoparticles on cells is essential. The aim of the following study is therefore to evaluate the cytotoxicity of magnetic ($Fe_3O_4$) and gold-coated magnetic nanoparticles ($Fe_3O_4$@Au) on various cell lines in order to clarify the risk of these materials for human use. Toxicity of these nanoparticles on human dermal fibroblasts (SKIN), human squamous cell carcinoma cells (A431 cells) and human epidermal keratinocytes (HaCaT cells) were determined using the MTT assay. Results showed that, within the used concentration range, $Fe_3O_4$ nanoparticles had no significant effect on all investigated cell lines, while $Fe_3O_4$@Au nanoparticles seem to have a moderate toxicity on all cell lines with some selectivity for the malignant cells, although it is yet moderate. The different characteristic of the cell lines' survival with respect to incubation time and nanoparticle concentration could be partly due to different cell death modes. Therefore, the prepared $Fe_3O_4$ nanoparticles are harmless and could be applied safely for skin cancer treatment or diagnosis.

*Keywords*: Magnetic nanoparticles; gold-coated; cytotoxicity; human skin cell lines; apoptosis.

## 1. Introduction

Major research efforts are undertaken to improve the selectivity and efficacy of anticancer agents to increase the quality of life for cancer patients. Nanomaterials are considered as one of the new modalities that could be used to overcome problems with existing anticancer therapies such as their lack of tumor selectivity. Among these nanomaterials, magnetic nanoparticles (MNPs) have received great attention.

MNPs have controllable sizes ranging from a few up to tens of nanometers, which place them at dimensions that are smaller than or comparable to those of cellular organelles.[1] Magnetic properties and pharmacologic distribution of magnetic particles depend strongly on their size.[2] It was found that particles ranging from 10 nm to 100 nm are optimal for subcutaneous injection and show the most prolonged blood circulation times. The particles in this size range are small enough to penetrate the very small capillaries within the tissues and, therefore, may offer the most effective distribution in certain tissues.[3]

Potential biomedical applications of MNPs would be hyperthermia, magnetic drug targeting, enhanced resolution magnetic resonance imaging, tissue repair, cell and tissue targeting and transfection.[4–8]

One of the most promising applications of MNPs is hyperthermia which is considered as a noninvasive method of cancer treatment; this is feasible via the use of magnetic field-induced excitation of MNPs.[9] Moreover, magnetic iron nanoparticles have been recognized as a promising tool for the site-specific delivery of drugs and diagnostic agents.[10,11]

MNPs possess adequate magnetic strength on their surface,[2] so that they can be transported by electrical field effects to the desired site and once the external magnetic field is removed, they can remain at the target site for a certain time period.[12] Because of that, MNPs are useful in drug delivery where they increase the selectivity of a drug and thus minimize undesirable side effects and toxicity.

These approaches necessitate the improvement of magnetic strength of nanoparticles and one of the logical methods used is to design their surface in a way that increases their magnetic efficiency.[13]

Metallic nanostructures, such as half nanoshells, have previously been developed as a platform for surface-enhancing properties. Iron core–gold shell nanoparticles were developed a decade ago to improve the magnetic susceptibility of iron oxide-based nanoparticles.[14] In addition, gold nanoshells could enhance the photothermal properties of tumor tissue by exposing nanoparticles to radiation near their plasmon-resonant absorption band producing local heating of tumor cells without harming the surrounding healthy tissues.[15] Iron oxide MNPs were already used for detection of nonpalpable lesions in breast cancer in animal studies.[16] Although many studies have been performed to assess the toxicity of nanoparticles on different cellular systems,[17–19] the published data have to be still considered insufficient to gain full understanding of the potential toxicity of these nanoparticles.

The objective of this work was therefore to study the cytotoxicity of both, uncoated magnetic and gold-coated MNPs on three human skin cell lines — one of these malignant — to estimate the risk of these materials for a possible use in human skin.

## 2. Material and Methods

### 2.1. *Preparation of magnetic ($Fe_3O_4$) nanoparticles*

MNPs were prepared photochemically as described previously in detail.[20] Shortly, preparation was done by dissolving a mixture of ferrioxalate with $FeCl_3 \cdot 6H_2O$ in 100 mL of 10% aqueous hydrogen peroxide solution with stirring. The pH of the solution was adjusted to 13 and the precipitates were collected by a magnet and washed several times with distilled water before air-drying them.

### 2.2. *Preparation of gold-coated magnetic ($Fe_3O_4$@Au) nanoparticles*

Gold-coated magnetic ($Fe_3O_4$@Au) nanoparticles were prepared as described previously.[15] About 0.01 mg of the prepared MNPs were suspended in 25 mL of glycerol and heated to 200°C with continuous stirring. About 5 mL of chlorauric acid ($HAuCl_4$) solution [$5 \times 10^{-3}$ M] was added dropwise to the mixture under continuous stirring for 15 min.

### 2.3. *Characterization of nanoparticles*

The prepared nanoparticles were characterized by UV-visible spectrophotometer, Thermo scientific, (USA). The X-ray diffraction (XRD) data of the prepared nanoparticles was collected at room temperature on an X-ray diffractometer (Rigaku, Japan) using CuK$\alpha$ radiation. Morphology of the prepared

nanoparticles was examined on high-resolution transmission electron microscopy (TEM "JEOL-100S", Japan). The detailed results of the prepared nanoparticles were discussed previously.[14,15]

### 2.4. *Cell cultures*

Primary human dermal fibroblasts (SKIN), human squamous cell carcinoma cells (A431 cells (ATCC: CRL-1555)) and human epidermal keratinocytes (HaCaT cells) were used. All cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, Vienna, Austria) supplemented with 10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (HEPES), 4 mM L-glutamine, 1 mM Na-pyruvate, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 5% fetal calf serum (FCS) (all from PAA-laboratories, Linz, Austria), in a humid atmosphere at 37°C and 7.5% $CO_2$. For experimental purposes, cells were cultured in 96-well plates (100 $\mu$L of cell suspension/well). Cells were allowed to attach for 24 h before treatment.[15]

### 2.5. *Cytotoxicity of nanoparticles*

Cell monolayers were washed with PBS and nanoparticles prepared in growth media were applied in different concentrations. Cytotoxicity of $Fe_3O_4$ and $Fe_3O_4$@Au nanoparticles was studied using concentrations ranging from 10 $\mu$g/mL up to 500 $\mu$g/mL and after different incubation times (24 h and 48 h). After incubation media containing nanoparticles were removed, the cells were washed with PBS and incubated for 24 h. Cell washing was done with extra care to insure the complete removal of nanoparticles to avoid inaccuracies due to light absorption and scattering of the prepared nanoparticles. Then, cell proliferation was determined by using the MTT assay (see below).

### 2.6. *MTT assay*

Mitochondrial dehydrogenase activity is an indication for the metabolic and therefore living state of cells. It was measured via the MTT assay as described previously,[21] but slightly modified. Briefly, cells were incubated for 45 min with 0.5 mg/mL MTT added to the culture medium. After that, the supernatant was aspirated and the resultant formazan was dissolved by addition of 100 $\mu$L DMSO–glycine (6 vol. dimethylsulfoxide + 1 vol. 100 mM glycine, pH 10) per well. Absorbance was read at 565 nm on a Spectrafluor microplate reader (Tecan, Austria). All assays were carried out in triplicate. After subtraction of the blank values, the mean values and standard deviation (SD) were calculated. Data are presented as percent activity of untreated control cells.

### 2.7. *Apoptosis detection (nuclear fragmentation)*

Nuclear fragmentation and chromatin condensation as results of apoptosis execution were evaluated by flow cytometric analysis of the cell cycle via measuring the DNA content of ethanol-fixed, ribonuclease A-treated and propidium iodide-stained cells after an incubation of 48 h with the prepared nanoparticles. Events showing lower fluorescence intensities than those of the $G_1$ peak (but higher than debris) were considered as the apoptotic fraction ("sub-$G_1$ area").[22,23] Experimental details are explained by Oberdanner *et al.*[24] For each sample, 10 000 events were analyzed using a FACSCanto II flow cytometer (BD Biosciences, Schwechat, Austria). Raw data for the distribution of DNA content were expressed as percentages in each cell cycle phase calculated by setting all events to 100%.

## 3. Results and Discussion

Both magnetic ($Fe_3O_4$) nanoparticles and gold-coated magnetic ($Fe_3O_4$@Au) nanoparticles were prepared as described previously.[15] Nanoparticle size, shape and uniformity were characterized by TEM and XRD analysis. The TEM image of the prepared MNPs ($Fe_3O_4$) demonstrates that the particles have spherical shapes and their size was in the range of $54 \pm 3$ nm while the TEM image of the prepared gold-coated MNPs ($Fe_3O_4$@Au) with different sizes was in the range of 22–55 nm (see Fig. 1, left).

The XRD spectra of the prepared nanoparticles are shown in Fig. 1, right. MNPs ($Fe_3O_4$) show six characteristic peaks for $Fe_3O_4$ at 220, 311, 400, 422, 511 and 440, as obtained previously[15] revealing that the resultant particles are in pure state. The XRD spectra of the prepared gold-coated MNPs ($Fe_3O_4$@Au) showed the characteristic diffraction peaks of $Fe_3O_4$ in addition to the characteristic diffraction peaks of gold at 111, 200, 220 and 310 (see Fig. 1, right).

In order to investigate the potential use of either uncoated MNPs ($Fe_3O_4$) or gold-coated MNPs

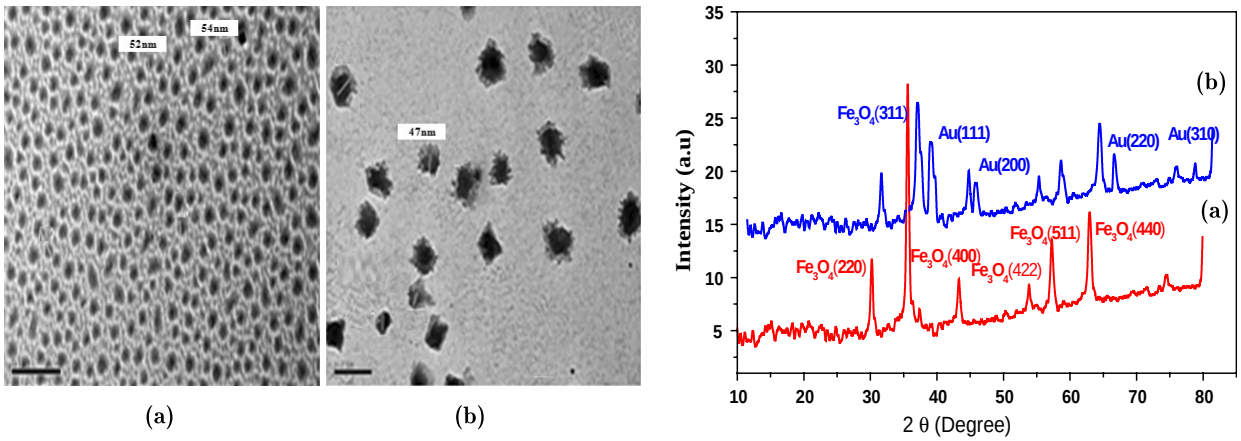


Fig. 1. TEM (left) images and XRD patterns of (right) (a) magnetic ($Fe_3O_4$) nanoparticles, (b) gold-coated magnetic ($Au@Fe_3O_4$) nanoparticles.[15]

($Fe_3O_4$@Au) in human skin, such as in cancer diagnosis and treatment, cytotoxicity was studied on different skin cell lines (primary human dermal fibroblasts: SKIN cells; human squamous cell carcinoma cells: A431 cells and human epidermal keratinocytes: HaCaT cells) using the MTT assay. The latter has proved to be of value for probing the toxicity of nanoparticles as it allows rapid evaluation of cell viability and growth, and exhibits good reproducibility.[25]

Cytotoxicity of nanoparticles was studied with different concentrations ranging from 10 $\mu$g/mL up to 500 $\mu$g/mL and for different incubation times (24 h and 48 h). Data are presented in percentages of cell survival in relation to the untreated control.

Results showed that $Fe_3O_4$ nanoparticles had almost no effect on cell viability of SKIN, A431 and HaCaT cells after incubation for 24 h and/or 48 h and up to 500 $\mu$g/mL [see Figs. 2(a), 3(a) and 4(a)].

A moderate decrease of survival after longer incubation time was only found in HaCaT cells. However, according to Mahmoudi *et al.*[12] iron oxide nanoparticles reducing cell viability for less than 20% can be considered as being biocompatible.

Naqvi *et al.*, found that the MTT assay of murine macrophage cells incubated with iron oxide

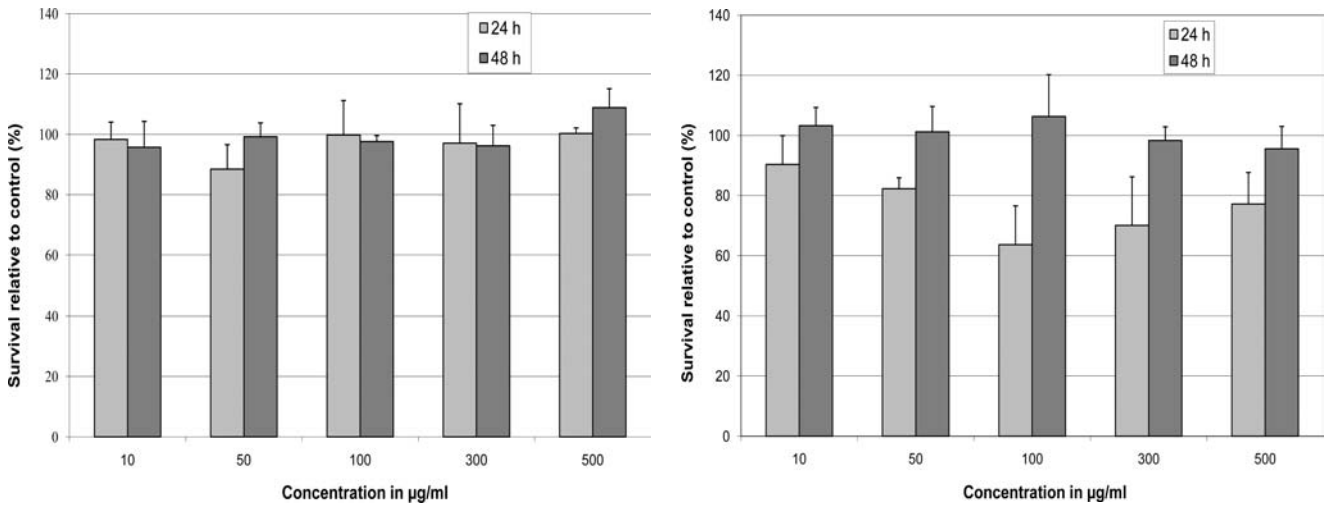


Fig. 2. Survival of SKIN cells treated with different concentrations of nanoparticles. SKIN cells incubated with different concentrations of nanoparticles; (a) uncoated magnetic ($Fe_3O_4$) nanoparticles; (b) gold-coated magnetic ($Fe_3O_4$@Au) nanoparticles. Cell viability was determined 24 h after an incubation of 24 h or 48 h and measured by the MTT assay. Data represent mean ± SD of three independent experiments.

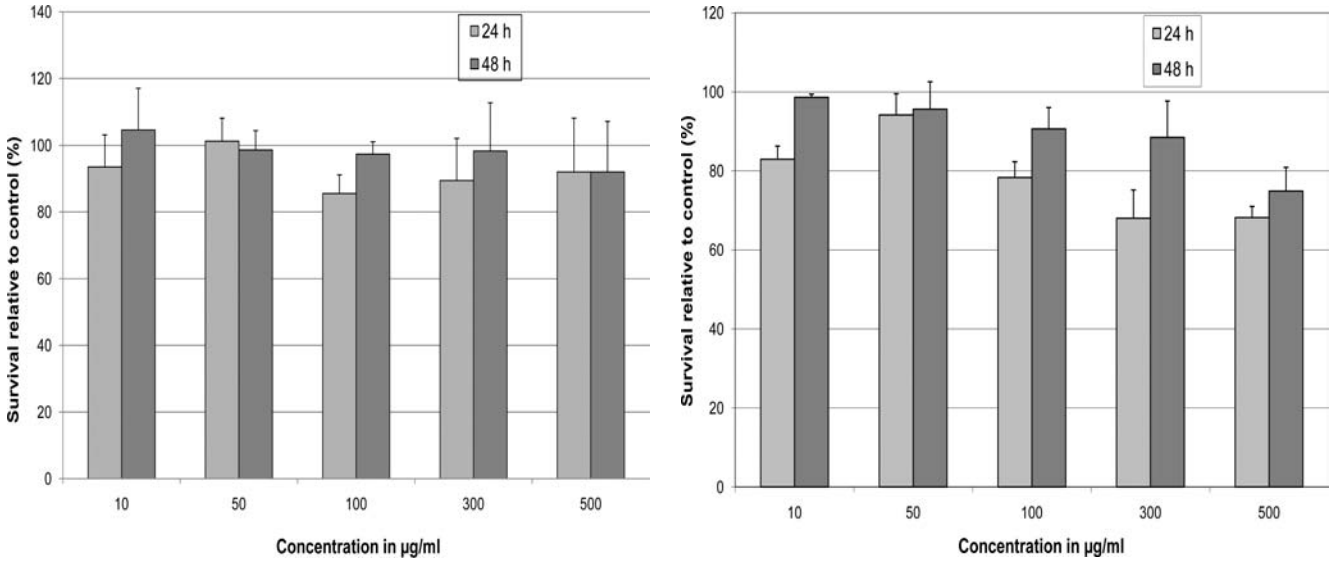


Fig. 3. Survival of A431 cells treated with different concentrations of nanoparticles. A431 cells incubated with different concentrations of nanoparticles; (a) uncoated magnetic ($Fe_3O_4$) nanoparticles; (b) gold-coated magnetic ($Fe_3O_4$@Au) nanoparticles. Cell viability was determined 24 h after an incubation of 24 h or 48 h and measured by the MTT assay. Data represent mean ± SD of three independent experiments.

nanoparticles in the size range of 30 nm showed 95% viability of cells in lower concentrations (up to 200 $\mu$g/mL), whereas at higher concentrations (300–500 $\mu$g/mL) and prolonged (6 h) exposure, viability reduced to 55–65%.[26] This difference in viability between their and our findings could be due to the time of MTT assay application; they washed cells and added MTT reagent directly after incubation with nanoparticles while in the current study, cells were washed with PBS and incubated for 24 h before applying MTT assay giving cells enough time to proliferate even at higher concentrations up to 500 $\mu$g/mL.

The low toxicity of iron oxide nanoparticles could be based on the following facts: the major oxidation

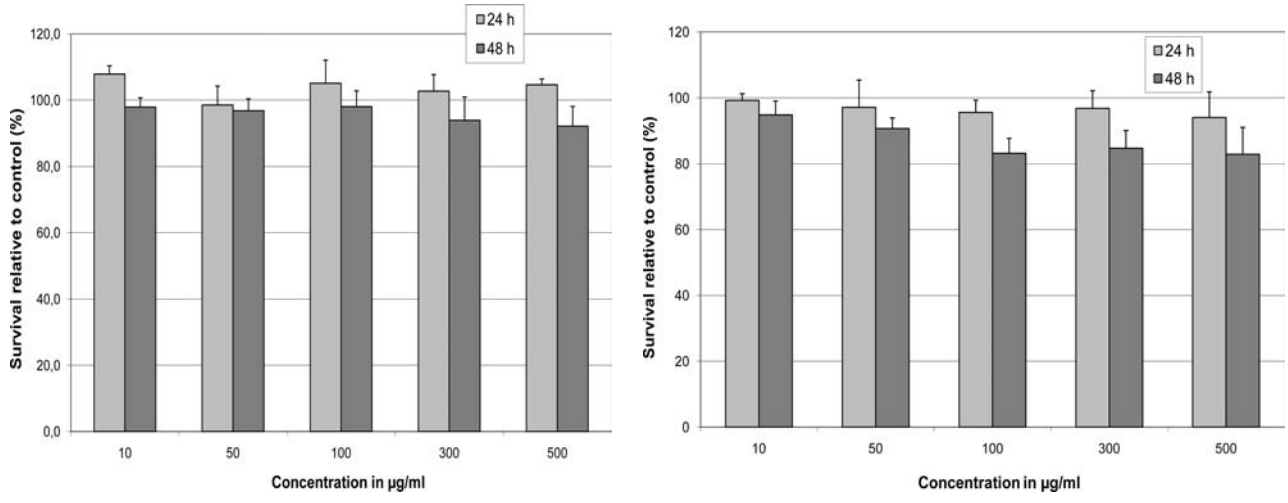


Fig. 4. Survival of HaCaT cells treated with different concentrations of nanoparticles. HaCaT cells incubated with different concentrations of nanoparticles; (a) uncoated magnetic ($Fe_3O_4$) nanoparticles; (b) gold-coated magnetic ($Fe_3O_4$@Au) nanoparticles. Cell viability was determined 24 h after an incubation of 24 h or 48 h and measured by the MTT assay. Data represent mean ± SD of three independent experiments.

products of iron in water, which is the main constituent of the cell, are iron oxides and hydroxides. As iron oxide is one of the most relevant trace elements needed for organisms to sustain life, iron oxide nanoparticles seem to be safe for biomedical use.[27]

Our finding is in agreement with Jain *et al.*, who stated that MNPs did not cause long-term changes in the liver enzyme levels and did not induce oxidative stress. Thus, they can be safely used for drug delivery and imaging applications.[28] In addition it was published that for MNPs made of iron oxide, no toxicity was observed.[29]

Analysis of cytotoxicity of $Fe_3O_4$@Au nanoparticles on SKIN cells after an incubation for 24 h showed that the number of living cells obviously decreases at higher concentrations: up to 23% of cells seem to be affected at a concentration of 500 $\mu$g/mL and about 35% at a concentration of 100 $\mu$g/mL. Interestingly, SKIN cells could recover after an incubation time of 48 h as no reduction of cell viability was detected [see Fig. 1(b)].

Likewise, analysis of cytotoxicity of $Fe_3O_4$@Au nanoparticles on A431 cells after an incubation for 24 h showed that already a concentration of 10 $\mu$g/mL reduces the viability for almost 20%; increasing concentrations of $Fe_3O_4$@Au nanoparticles induced a slight further decrease in the number of living cells with up to 32% of the cells being killed at a concentration of 500 $\mu$g/mL. Similar to SKIN, the A431 cells could also recover by increasing the incubation time to 48 h, but only partly. About 26% of the A431 cells were still killed at the highest concentration [see Fig. 2(b)]. Also here, it is likely that some cells are killed by nanoparticle treatment for 24 h and that the surviving fraction activates proliferation during the subsequent 24-h treatment period. Although the toxicity of $Fe_3O_4$@Au nanoparticles is higher on the malignant A431 cells than on the normal SKIN cells after 48 h of incubation, it is yet moderate.

Cytotoxicity of $Fe_3O_4$@Au nanoparticles on HaCaT cells after incubation for 24 h showed no major effect on cell viability even at a concentration of 500 $\mu$g/mL. However, by increasing the incubation time to 48 h, a moderate decrease in the number of living cells can be observed as up to 18% of cells were killed at the highest concentration [see Fig. 3(b)], an effect similar to treatment with $Fe_3O_4$ nanoparticles.

Therefore, SKIN and A431 cells seem to have some kind of adaptive repair mechanism, which initiates a proliferation stimulus in the surviving cells compensating for cell kill after longer incubation times. In contrast, HaCaT cells, which have a defect in their p53 repair system, rather accumulate the damage. p53 has multiple biological functions which include control of gene expression, modulation of cellular repair and thus regulation of cell proliferation.[30] Our findings are in agreement with Li *et al.*,[31] who showed safety and biocompatibility of $Fe_3O_4$@Au nanoparticles *in vitro* (no increase of cytotoxicity and micronuclei in L929 mouse fibroblasts) as well as *in vivo* in mice and dogs.

From the gained data, it can be deduced that after a 48-h incubation period with the prepared nanoparticles, $Fe_3O_4$nanoparticles induced no relevant toxicity on both normal and malignant cells [see Figs. 2(a), 3(a) and 4(a), while $Fe_3O_4$@Au nanoparticles showed moderate toxicity at higher concentrations starting from 100 $\mu$g/mL on the p53-deficient HaCaT and on the malignant A431 cells but not on the normal SKIN cells [see Figs. 2(b), 3(b) and 4(b)]. The moderately different reactions of the cells toward $Fe_3O_4$@Au nanoparticles could be due to different cellular uptake of various cell types. Moreover, differences in pH levels and redox status of ions within malignant and immortalized cells in contrast to normal cells could induce such a difference.[19]

Discrimination between apoptosis and necrosis resulting from the cytotoxic action of $Fe_3O_4$@Au nanoparticles was achieved via a nuclear fragmentation assay, the measurement of the sub-$G_1$ peak in cell cycle analysis using flow cytometry. Results show an increasing amount of the SKIN cell population in the sub-$G_1$ phase after incubation with $Fe_3O_4$@Au nanoparticles for 48 h in a dose-dependent manner [see Fig. 5(a)], although there is no increase in overall cell death observed after the same concentrations and incubation period [see Fig. 2(b)]. The decrease in the number of living SKIN cells after 24-h incubation with $Fe_3O_4$@Au nanoparticles could be due to apoptosis; the remaining surviving cells enhance their proliferation after 48 h to compensate cell death.

The data show furthermore that there are no changes in sub-$G_1$ phases between untreated A431 cells and those treated with different concentrations up to 500 $\mu$g/mL of $Fe_3O_4$@Au nanoparticles [see Fig. 5(b)]. Therefore, the moderate cell killing of

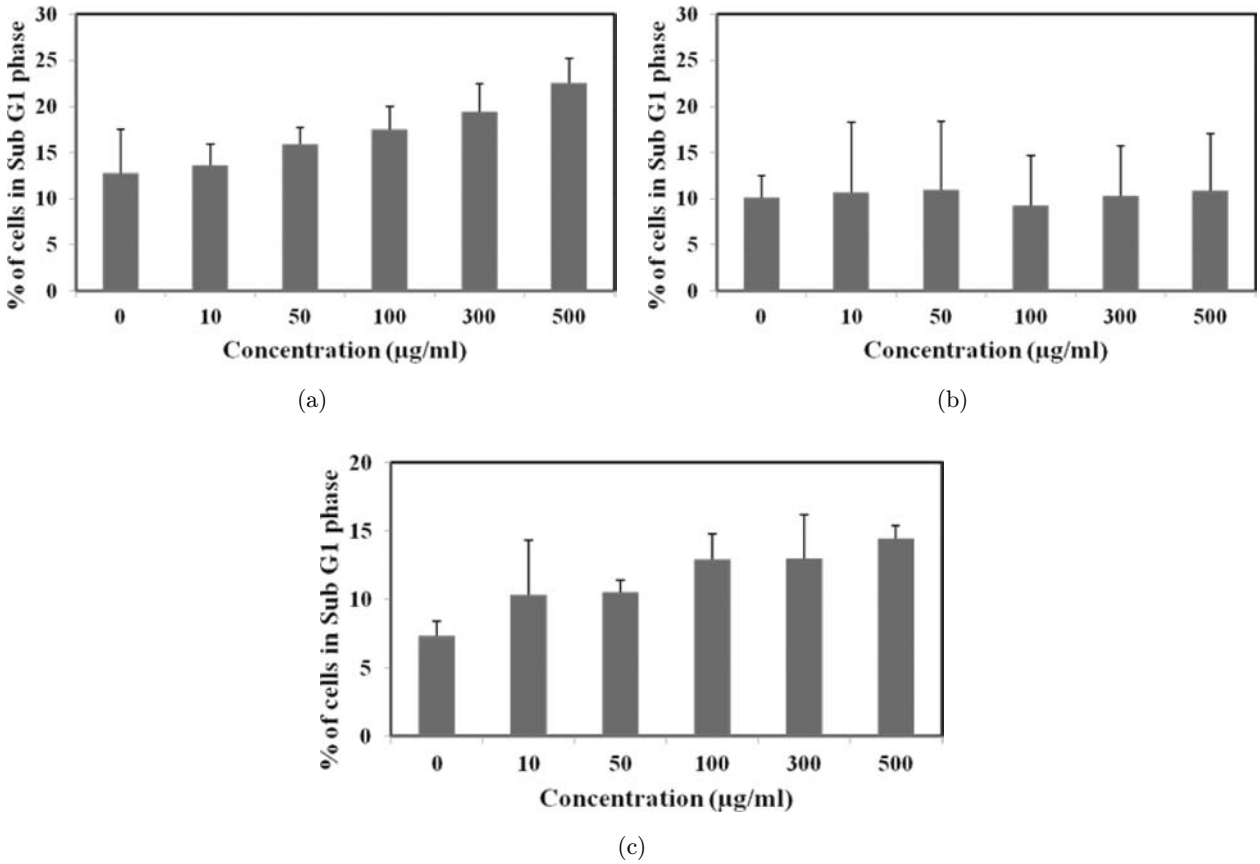


Fig. 5. Apoptosis determination by flow cytometric analysis via sub-$G_1$ assay. (a) SKIN cells; (b) A431 cells and (c) HaCaT cells. Cells were harvested after a treatment of 48 h with different concentrations of gold-coated magnetic ($Fe_3O_4$@Au) nanoparticles. Data represent mean ± SD of three independent experiments.

A431 cells after 48 h of incubation with 100 $\mu$g/mL to 500 $\mu$g/mL of $Fe_3O_4$@Au nanoparticles [see Fig. 3 (b)] seems to take place by necrosis. Also here it is likely that up to 32% of the A431 cells are killed by $Fe_3O_4$@Au nanoparticle treatment within 24 h and that the surviving cells increase their proliferation rate during the subsequent 24-h treatment period to compensate partly for loss of cells.

Moreover, data show a moderate increase in sub-$G_1$ phases of HaCaT cells as the concentration of $Fe_3O_4$@Au nanoparticles increases [see Fig. 5(c)]. Since, at the same incubation time, a slight cytotoxicity of $Fe_3O_4$@Au nanoparticles on HaCaT cells could be found in a dose-dependent manner [see Fig. 4(b)], $Fe_3O_4$@Au nanoparticles are likely to induce cytotoxicity by promoting HaCaT cell death via apoptosis.

There is some evidence that a nanoparticle-induced reactive oxygen species (ROS) oxidant stress response might be the major mechanism for induction of various biological effects.[32] A recent study demonstrated that exposure to a higher concentration of nanoparticles resulted in enhanced ROS generation, leading to cell injury and death. The cell membrane injury induced by nanoparticles revealed loss of the majority of the cells by apoptosis.[26]

Finally, we can state that $Fe_3O_4$ nanoparticles had no major effect on cell viability of both malignant cells and normal cells up to a concentration of 500 $\mu$g/mL. $Fe_3O_4$@Au nanoparticles seem to have a moderately toxic effect. Generally, the mode of cell death may differ according to the uptake characteristics of nanoparticles in various cell types. This could be the reason for the differences in the cell lines' survival with respect to incubation time and nanoparticle concentration. It can be concluded that within the investigated concentration range, the use of $Fe_3O_4$ nanoparticles is harmless while the use of $Fe_3O_4$@Au nanoparticles is moderately toxic.

In general, our research is a primary step toward developing an integrated nanomedicine, which

concentrates in the present work on the study of cytotoxicity of two different types of MNPs on human skin cells. Based on our findings, it cannot be excluded that $Fe_3O_4$ nanoparticles will be applicable in skin cancer treatment and nonmalignant skin indications such as wound healing or cosmetic improvement.

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