## ORIGINAL ARTICLE

# *In vitro* effect of hyperthermic Ag and Au $Fe_3O_4$ nanoparticles in cancer cells

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## Summary

**Purpose:** *To investigate the anti-cancer efficacy of hyperthermic Ag and Au $Fe_3O_{4\,core}$ nanoparticles via cytotoxicity study (MTT assay) and the underlying molecular mechanism of action (changes in gene expression via quantitive real time PCR (qRT-PCR).*

**Methods:** *HEK293, HCT116, 4T1 and HUH7 human cell lines and 4T1 musculus mammary gland cell line were incubated with $Fe_3O_{4\,core}$ Ag(Au)$_{shell}$ nanoparticles (NPs) prior to a hyperthermia session. MTT assay was performed to estimate the cytotoxic effects of these NPs. RNA extraction and cDNA synthesis followed so as to quantify mRNA fold change of hsp-70, p53, bcl-2 and casp-3 via qRT-PCR.*

**Results:** *$Fe_3O_{4\,core}$ Au$_{shell}$ (concentrations of 400 and 600µg/mL) produced the greatest reduction of viability on HCT116 and 4T1 cells while $Fe_3O_{4\,core}$ Ag$_{shell}$ (200, 400 and 600µg/mL) reduce viability on HUH7 cells. Hsp-70, p53 and casp-3 were up-regulated while bcl-2 was downregulated in most cases.*

**Conclusions:** *$Fe_3O_{4\,core}$ Ag(Au)$_{shell}$ induced apoptosis on cancer cells (HCT116 and HUH7) via the p53/bcl-2/casp-3 pathway. 4T1 cells also underwent apoptosis via a p53-independent pathway.*

**Key words:** *anti-tumour effect, $Fe_3O_{4\,core}$ Ag(Au)$_{shell}$ nanoparticles, hyperthermia, magnetic nanoparticles, tryptophan*

## Introduction

Hyperthermia refers to the type of treatment in which body tissue is exposed to high temperature in order to damage and kill cancer cells or make them more sensitive to radiation and anticancer drugs [1,2]. Different tools have been suggested to induce hyperthermia; ionizing radiation, laser and microwaves to heat up malignant tissues. Radiotherapy and chemotherapy have been widely used in tumour regions but leading to harmful effects on healthy tissues [2,3].

Nanotechnology introduced non-invasive techniques using different nanoparticles (NPs) as heating mediators. Gold and silver NPs can be applied on their own or in combination with other molecules (i.e., polymers, surfactants, organic dyes) for targeting, imaging and therapeutics [4]. Hyperthermic NPs are a promising tool in cancer therapy. In magnetic hyperthermia, NPs like $Fe_3O_4$ NPs, absorb energy and convert it into heat (>41.5°C). Magnetic NPs have been successfully used in many studies and even progressed in clinical trials [5,6]. However, in order to succeed a specific anti-tumour effect to the tumour site, the construction of targeted NPs seems compulsory.

Previously, we estimated the nanotoxicity of colloidal mono- and bimetallic silver/gold NPs stabilized with tryptophan (Trp), in three cell lines 4T1, a breast cancer cell line, HCT116, a colon cancer cell line and HEK293, embryonic kidney cells [7]. We found that the NP toxicity was lower in non-cancer cells, making them promising tools for cancer treatment approaches [7,8]. In this study we investigated the effect of hyperthermic Fe$_3$O$_{4\,core}$ Ag(Au)$_{shell}$ NPs in cancer cell cultures. Our initial hypothesis was that cancer cell lines exposed to hyperthermic NPs will suffer thermal damage which will lead to cellular death. In order to test this hypothesis, we included a fourth cell line, HUH7, a well-differentiated hepatocyte derived cellular carcinoma and we approached the underlying molecular mechanism *in vitro*, in cancer cells. We estimated the expression of genes involved in programmed cell death (casp-3, p53, bcl-2) and hsp-70, a gene for cellular responses to environmental stressors including hyperthermia [9]. These NPs are stabilized with Trp as an effective way in attenuating potential hepatotoxicity and nephrotoxicity of NPs during their future *in vivo* application [10].

## Methods

### Fe$_3$O$_{4\,core}$ Ag(Au)$_{shell}$ NPs synthesis

Colloidal solutions of nanocomposites containing iron oxide core and noble metal shell Fe$_3$O$_{4\,core}$ Ag(Au)$_{shell}$ were obtained via chemical reduction of metal ions (AgNO3, HAuCl4, Merck, Germany) by amino acid tryptophan (Trp, SC12-20120713, China) in the presence of magnetic fluid - suspension of iron oxide Fe$_3$O$_4$ in sodium oleate. The synthetic procedure was similar to the previously described [7]. Initial solution of Trp was adjusted to high pH and heated to boiling. Then, magnetite was injected followed by silver nitrate (tetrachlorauric acid). The components molar ratio was v(Trp): v(M): v(Fe$_3$O$_4$) = 2:1:0,5. Colloid was stirred and heated continuously.

### Cell culture

HEK293, HCT116, 4T1 and HUH7 cell lines were grown in DMEM high glucose culture medium (BioSera, Nuaille, France) containing 10% fetal bovine serum (FBS), 2 mmol/L glutamine, 100 U/ml penicillin and 100 g/ml streptomycin at 37°C. The medium was changed every 48 h and cells were passaged once weekly using standard trypsin-EDTA concentrations. Beginning at 38, 32, 41 and 36 cell passages respectively, cells were cultured continuously. Cells were frozen in freezing medium containing FBS, 5% DMSO. All cell lines used were adherent. HEK293 cell line was used as control group (non-cancer cell line) in our experiments.

### MTT assay

The MTT cell viability assay measures alterations in cell viability; when metabolic events lead to apoptosis or necrosis, the cell viability is decreased. As a general protocol, 50.000 cells/well were seeded in 24-well plates (Corning-Costar, Corning, NY) and cultured overnight. Positive, negative and background controls were used throughout the study. Positive control had cells with culture medium but not exposed to NPs. Negative control had NPs without cells. Background control had culture medium without cells. The cell lines used were treated with 200, 400 and 600 µg/mL of Fe$_3$O$_{4\,core}$ Ag$_{shell}$, Fe$_3$O$_{4\,core}$ Au$_{shell}$ and Fe$_3$O$_4$ alone for 1 h and then ionized for 15 min. Subsequently, the cells were rinsed once and incubated in 37°C with 100µL of serum-free medium containing 0.5 mg/mL MTT. After 1.5h, 100µL of SDS-HCl were added to each well, mixed thoroughly and incubated for 1 h at 37°C. Optical densities (OD) were read at 570 nm (reference filter was set at 690 nm), using a microplate spectrophotometer (SPECTROstarNano, BMG LABTECH, Ortenber, Germany). Absorbances were normalized with respect to the untreated control cultures to calculate changes in cell viability.

### Hyperthermia

The hyperthermia session was performed using a water loaded circular waveguide applicator (7 cm diameter) with an effective aperture of 7 cm [11]. HEK293, HCT116, 4T1 and HUH7 cells were incubated for 20 min at 43°C. The hyperthermia device operated as a 433 MHz microwave heating. The device had an omitted power of 100 Watts RMS. However, the transmitted power in our case was at the level of 15-20 Watts for 4 min [12].

### RNA extraction and quantitative real-time RT-PCR

Total RNA extraction (for all three NPs used and for concentration of 400µg/mL) was performed using TRIzol reagent (Thermo Fisher Scientific), according to the manufacturer's instructions. Reverse transcription was performed using the PrimeScript First Strand cDNA Synthesis Kit (TAKARA). The reaction conditions were as follows: 37°C for 30min and 85°C for 5s. The reaction was performed on Thermal Cycler (Kyratec Super Cycler). Assessment of casp-3, bcl-2, p53 and hsp-70 mRNA levels was performed for HCT116, 4T1, HUH7 and HEK293; 4T1 cells are p53 null [13] and as such expression of p53 was not investigated.

Quantitative real-time RT-PCR was conducted on an ABI Prism 7000 apparatus (Applied Biosystems, Foster City, CA, USA). Each cDNA sample was mixed with specific primer sets and PCR master mix (KAPA SYBR FAST qPCR Kit). The levels of genes expression were normalized by subtracting the Ct value of the GAPDH RNA internal control from that of the GOI (gene of interest) ($\Delta$Ct=-|CtGOI-CtGAPDH|). To determine the relative expression of GOI in cancer cells compared to non-cancer cells the 2$\Delta\Delta$Ct model was used, where $\Delta\Delta$Ct=$\Delta$CtGOI-$\Delta$CtGAPDH.

### Statistics

All statistical analyses were performed using GraphPad version 3.00 (GraphPad Software, San Diego, CA). P<0.05 was considered statistically significant.

# Results

## *Characterization of magnetic NPs*

Prepared colloidal solutions of magnetic NPs containing iron oxide core and noble metal shell had a bright yellow and red colour inherent to NPs of silver and gold respectively.

A specific colour is a distinguishing feature of noble metal NPs and is caused by the phenomenon of localized surface plasmon resonance (LSPR) that appears as absorption in the visible range of the spectrum. The maxima of LSPR absorption bands of $Fe_3O_{4\,core}\,Ag_{shell}$ and $Fe_3O_{4\,core}\,Au$ shell colloids were localized at 420 and 527 nm (Figure 1), indicating the formation of continuous shell around the magnetite particles. The obtained NPs, with both silver and gold shell, were of spherical shape with the average size of 10-20 nm according to data obtained by transmission electron microscopy (TEM) and dynamic light scattering (DLS) methods (Figure 1). Both colloids carried neutral pH, namely 7.2 for $Fe_3O_{4\,core}\,Au_{shell}$ and 7.7 for $Fe_3O_{4\,core}\,Ag_{shell}$.

## *Hyperthermic-NPs induced toxicity*

$Fe_3O_{4\,core}\,Ag_{shell}$, $Fe_3O_{4\,core}\,Au_{shell}$ and $Fe_3O_4$ NPs were tested regarding their toxicity under hyperthermic and non-hyperthermic conditions (200, 400 and 600 μg/mL). The MTT results are shown in bar diagrams for HEK293 (Figure 2a), HCT116 (Figure 2b), 4T1 (Figure 2c) and HUH7 (Figure 2d) cells. The largest viability decrease was in HCT116

cells (400 μg/mL and 600 μg/mL of $Fe_3O_{4\,core}\,Au_{shell}$ NPs reduced viability at 40 and 55% respectively). $Fe_3O_{4\,core}\,Au_{shell}$ NPs appeared highly toxic for 4T1 cells. Non-ionized 4T1 cells showed a low, dose-independent toxicity (approximately 80% viability), while ionized cells showed a decrease of viability by 58 and 65% (for concentrations 400 μg/mL and 600 μg/mL). In HUH7 cells, there was 30% difference of viability between ionized and non-ionized cells incubated with $Fe_3O_{4\,core}\,Ag_{shell}$. (200, 400 and 600 μg/mL). Interestingly, in 400 and 600 μg/mL of hyperthermic $Fe_3O_{4\,core}\,Au_{shell}$ and in 200 and 400 μg/mL with $Fe_3O_{4\,core}\,Ag_{shell}$ the toxic effects of these NPs were reduced in non-cancer cells HEK293 (Figure 2a).

## *Effect of hyperthermic NPs on hsp-70 and apoptotic genes*

qPT-PCR was performed to analyse the mRNA levels of hsp-70, bcl-2 and casp-3 in HEK293, HCT116, 4T1 and HUH7 cells and p53 in HEK293, HCT116 and HUH7 (400 μg/mL of all three NPs tested). In HEK293 cells (Figure 3a), $Fe_3O_{4\,core}\,Au_{shell}$ led to the up-regulation of hsp-70, while bcl-2 was down-regulated. The $Fe_3O_4$ NPs slightly down-regulated p53 and bcl-2 expression, with no effect on hsp-70 expression. Casp-3 expression remained almost unchanged. In ionized HCT cells hsp-70, p53 and casp-3 were up-regulated in all three NPs, while bcl-2 was down-regulated (Figure 3b). Similarly, ionized 4T1 cells showed up-regulation of hsp-70 and casp-3, while bcl-2 was down-regulated in $Fe_3O_{4\,core}$
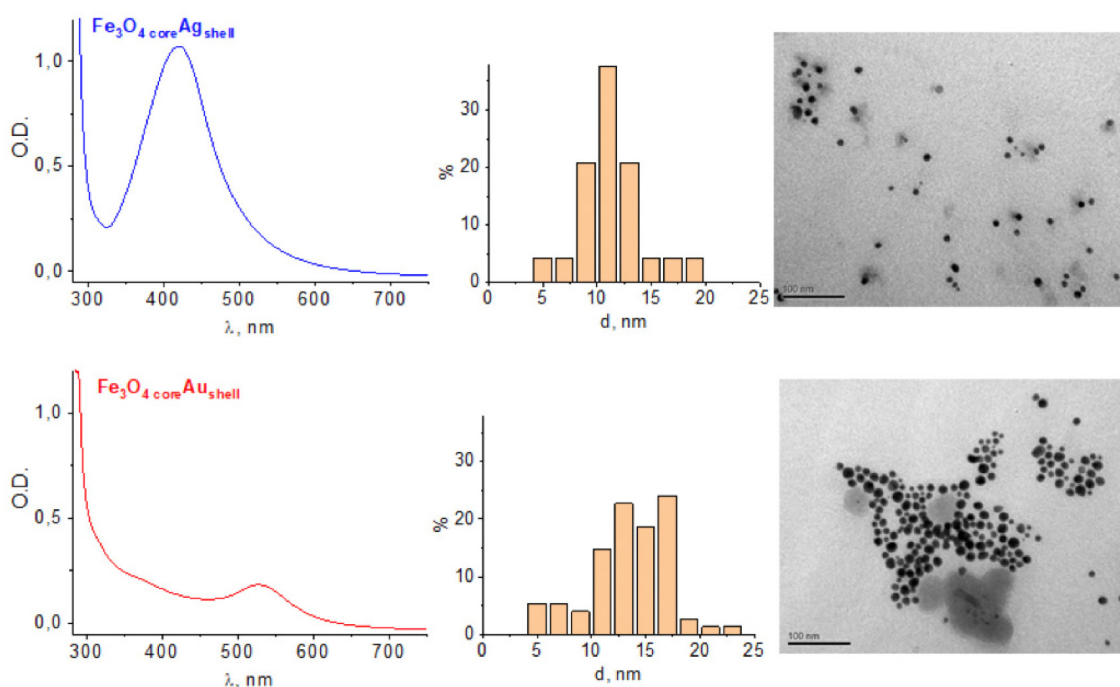


**Figure 1.** Properties of $Fe_3O_{4core}\,Ag(Au)_{shell}$ nanoparticles. Absorbance spectra of magnetic nanoparticles with the shell of silver (top) and gold (bottom) with corresponding TEM images and size distribution calculated with ImageJ.
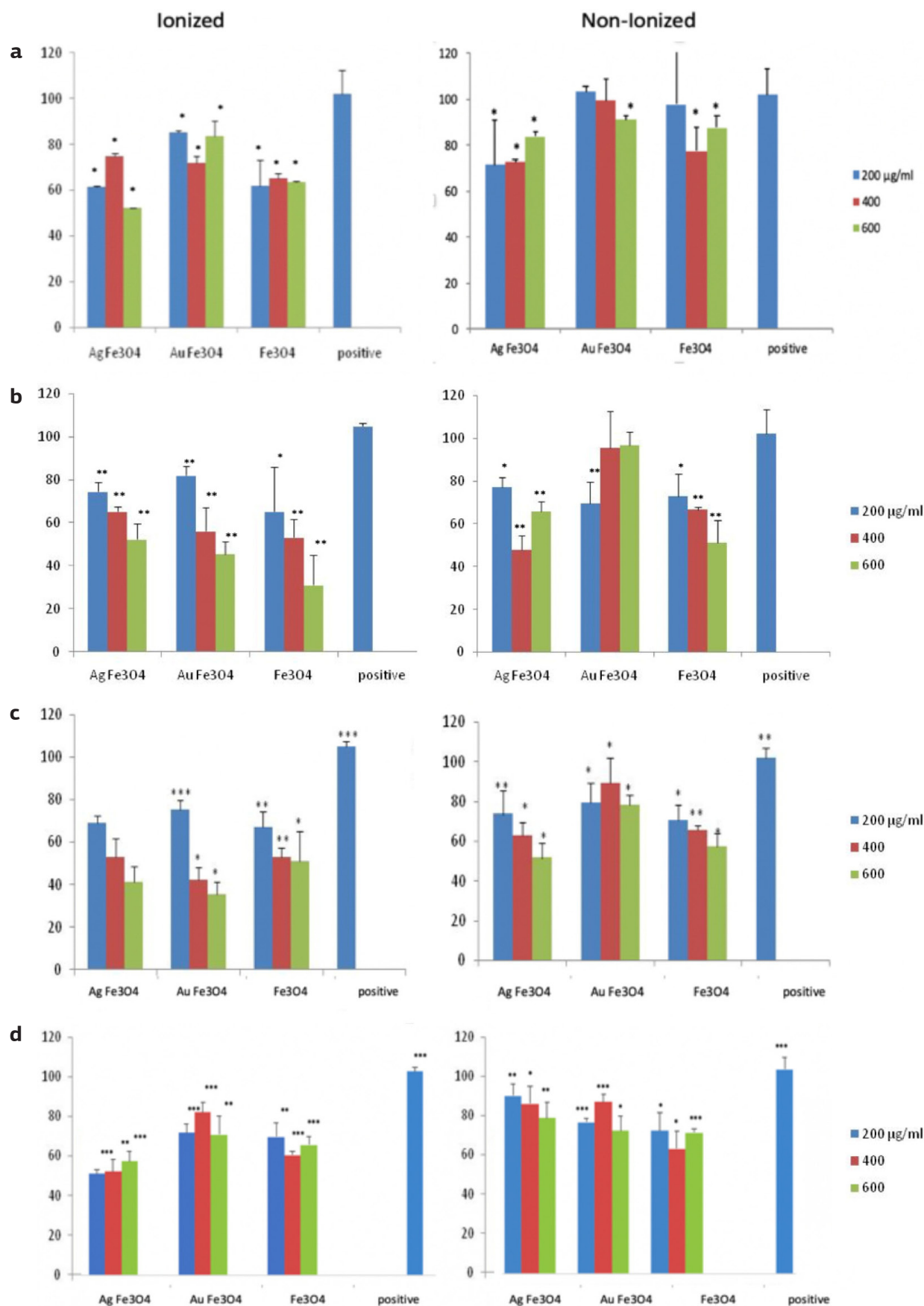
**Figure 2.** Viability percentages. Graphs of MTT assay after treatment with various NPs of different concentrations of Fe₃O₄ on HEK293 **(a)** and HCT116 **(b)**, 4T1 **(c)** and HUH7 **(d)** and subsequent ionization (graphs on the left). Non-ionized cells (graphs on the right) were used as controls. Positive control shows cells without Fe₃O₄ incubation. *p<0.05, **p<0.01, ***p<0.001.

Au $_{shell}$. p53 expression was not calculated since as already mentioned 4T1 cells are p53 null (Figure 3c). Finally, HUH7 cells showed an up-regulation of p53 and casp-3, while bcl-2 was down-regulated in $Fe_3O_{4\,core}$ Ag $_{shell}$. (Figure 3d).

## Discussion

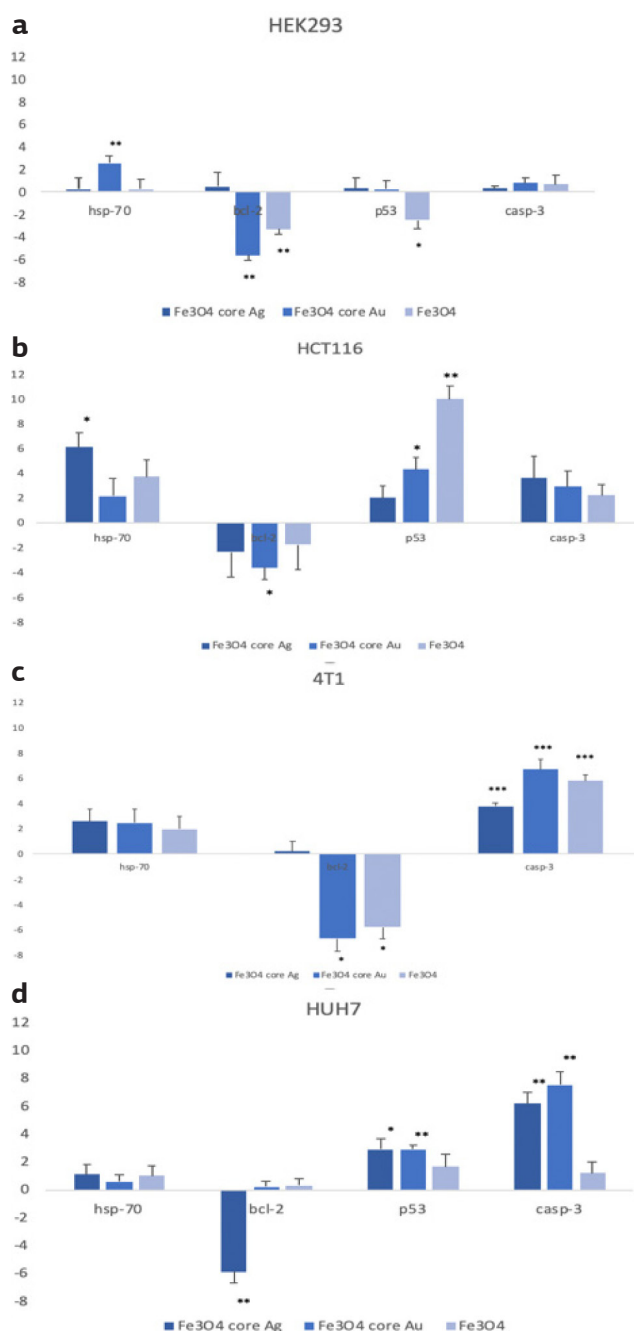According to our results, the NPs exhibit a toxic effect against cancer cells after ionization.

**Figure 3.** mRNA levels fold change. *p<0.05, **p<0.01, ***p<0.001. Fold change of hsp-70, bcl-2, p53 and casp-3 in ionized HEK293 **(a)**, HCT116 **(b)**, 4T1 **(c)** and HUH7 **(d)** cells with 400µg/mL of $Fe_3O_{4\,core}$ Ag $_{shell}$, $Fe_3O_{4\,core}$ Au $_{shell}$ and $Fe_3O_4$ nanoparticles.

$Fe_3O_{4\,core}$ Au $_{shell}$. This observation indicates that in concentrations 400 and 600 µg/ml of $Fe_3O_4$ NPs can work as hyperthermic NPs in both 4T1 and HCT 116 cells lines (Figures 2b,c). Specifically, non-ionized HCT116 show no toxicity in concentrations 400 and 600 µg/ml of $Fe_3O_4$ (~5% toxicity), while in 200 µg/ml of $Fe_3O_4$ the toxicity is ~30%. On the other hand, ionized HCT116 in concentrations 400 and 600 µg/ml of $Fe_3O_4$ showed ionization-dependent toxicity (40% and 55% respectively). In 200 µg/ml of $Fe_3O_4$ the toxicity is no ionization-dependent.

Regarding non-ionized 4T1, they show maximum 20% toxicity in the three concentrations tested, opposed to ionized 4T1 that in the concentrations 400 and 600 µg/ml of $Fe_3O_4$ show high toxicity (~58% and 65% respectively), indicating ionization-dependent toxicity. $Fe_3O_{4\,core}$ Ag $_{shell}$ NPs because of the toxic effect of Ag NPs on their own, compared to Au NPs that are considered to be the less toxic metal NPs for *in vivo* applications, do not seem to act as heating mediators [7,14]. $Fe_3O_{4\,core}$ Ag $_{shell}$ NPs show no ionization-dependent toxicity in both cell lines. Interestingly, in the same concentrations the toxic effect of hyperthermic $Fe_3O_{4\,core}$ Au $_{shell}$ NPs is minimized in non-cancer cells HEK293 (Figure 2a) VS ~55% viability in cancer cells with 400µg/ml and 45% viability with 600µg/ml) (Figures 2 a-c).

On the contrary, it is $Fe_3O_{4\,core}$ Ag $_{shell}$ NPs that show a decrease of viability in HUH7 cells, serving as hyperthermia-inducing agents whereas both $Fe_3O_{4\,core}$ Au $_{shell}$ and $Fe_3O_4$ have a lower cytotoxic effect. Ag NPs show a dose-independent toxicity with almost 30% difference between ionized and non-ionized cells in all three concentrations tested. Thus, it seems that there is not a single hyperthermic NPs suitable for all cancer cell types but different hyperthermic NPs are cytotoxic for different cancer cells. Furthermore, as already mentioned, Ag NPs have an endogenous toxicity that Au NPs lack. This finding suggests that hyperthermia and Ag could have a synergistic effect on HUH cells; neither non-ionized HUH7 cells incubated with $Fe_3O_{4\,core}$ Ag $_{shell}$ nor bare $Fe_3O_4$ on ionized HUH7 show similar toxicity. Therefore, these results indicate that HUH7 could be hyperthermia-resistant, requiring the action of Ag for toxicity to be induced. Indeed, increased resistance to hyperthermia has already been described in a study [15] in which integrin-linked kinase was associated with poor response to hyperthermia. However, the molecular basis of this phenomenon remains unclear.

We also examined the expression of hsp-70 and apoptosis-related genes (p53, casp-3 and bcl-2). Hsp-70 is a protein family induced under environmental stress, heat shock included, and assists the cell to cope with denatured proteins and prevents

apoptosis. Considered as the hallmark of hyperthermia, its up-regulation in exposed cells is found in several studies [16,17]. Hsp-70 serves as a danger signal by triggering immunological responsiveness against cancer cells and increasing tumour tissue infiltration by eosinophil granulocytes [18]. Tsang et al [19], reported that administration of both hsp-70 and dendritic cells at irradiated cancer tissue triggers a more potent anti-cancer response than dendritic cells alone. In our study, except Fe$_3$O$_{4\,core}$ Au$_{shell}$ NPs, ionized HEK293 showed almost no fold change in hsp-70 expression, suggesting a lower intake of hyperthermic NPs. On the other hand, HUH7 had a mild up-regulation of hsp-70. Combined with the MTT results, this finding supports the synergistic hypothesis (hyperthermia combined with Ag result in increased toxicity); hyperthermia alone mildly affected the viability of HUH7 cells. HCT116 and 4T1 cells showed the highest up-regulation of hsp-70 in all four cell lines and also showed a high decrease in viability (ionized HCT116 and 4T1 using Fe$_3$O$_{4\,core}$ Au$_{shell}$ NPs, 400µg/mL).

Following the study of hsp-70 fold change, our next goal was to approach the molecular pathway that leads to cancer cell death. Apoptosis is of crucial importance for cell fate since pro- and anti-survival signals determine tumour initiation and progression. However, cancer cells are apoptosis-resistant while many chemotherapeutic drugs function by triggering apoptotic mechanisms [20]. P53 is a tumour suppressing gene that guards genome and inhibits tumorigenesis since it promotes DNA repair and cell death [21]. Bcl-2 is anti-apoptotic gene, targeted by p53 transcription factor and promotes cell survival by binding cytochrome C residues. Casp-3 also has a key-role in apoptosis by catalysing the cleavage of several important intracellular proteins [22-24].

In our study, p53 and casp-3 were found up-regulated, except HEK293 cells where they remained almost unchanged for all NPs used. On the contrary, bcl-2 was found down-regulated in every cell line except Fe$_3$O$_{4\,core}$ Ag$_{shell}$ in HEK293 and Fe$_3$O$_{4\,core}$ Au$_{shell}$ and Fe$_3$O$_4$ HUH7 cells. These results are consistent with the MTT results; HEK293 had the mildest viability reduction and also showed the mildest fold change in gene expression. All the cancer cell lines which had an increased toxicity also showed an analogous fold change in gene expression. These results reveal that cancer cells treated with the hyperthermic NPs used in this study showed anti-cancer potency via the caspase cascade and the p53/bcl-2 apoptotic pathway. However, 4T1 cells are p53-null. Thus, the cellular death mechanism is triggered by a p53 independent pathway. Yerlikaya

et al [13], showed that a proteasome inhibitor leads to a p53 independent apoptosis in 4T1 p53-null cells. Despite the different stimuli (hyperthermia vs proteasome inhibition) the cellular response was the same: caspase-3 was up-regulated. The latter highlights that p53-deficient cell can also lead to apoptosis via caspase-3. However, further research in the underlying mechanisms involved is crucial since in some cancer types p53 is found mutated up to 50% [25]. Additionally, studies have shown contradicting results about the role of pro-apoptotic genes involved in p53-independent apoptosis [13].

Our results are in agreement with other studies in which hyperthermia (not mediated by NPs) results in apoptotic death of cancer cells [26,27], while two more studies using NP-mediated hyperthermia also support this conclusion [28,29]. Furthermore, one study using hyperthermia on p53-deficient H1299 lung cancer cells also showed that the underlying mechanism of cellular death is apoptosis [30].

Based on our findings, the NPs we used seem to have specificity against cancer cells. We deemed that is has to do with the use of tryptophan (Trp) as a stabilizer and reducing agent. Trp is demonstrated to have rather a positive effect on both cell lines compared to the positive control, especially on cancer cells (data not shown). Thus, the increased metabolism of Trp by cancer cells specifically may be beneficial in order to increase the anti-tumour effect of NPs [31]. However, further research is required to elucidate the p53-independent apoptosis triggered by hyperthermic NPs and to predict the vulnerability of cancer cell types towards a particular hyperthermic NP (Fe$_3$O$_{4\,core}$ Au$_{shell}$ or Fe$_3$O$_{4\,core}$ Ag$_{shell}$).

## Acknowledgements

## Conflict of interests

The authors declare no conflict of interests.

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