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Influence of the surface coating on the cytotoxicity, genotoxicity and uptake of gold nanoparticles in human HepG2 cells

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ABSTRACT: The toxicological profile of gold nanoparticles (AuNPs) remains controversial. Significant efforts to develop surface coatings to improve biocompatibility have been carried out. *In vivo* biodistribution studies have shown that the liver is a target for AuNPs accumulation. Therefore, we investigated the effects induced by ~20 nm spherical AuNPs (0–200 μM Au) with two surface coatings, citrate (Cit) compared with 11-mercaptoundecanoic acid (11-MUA), in human liver HepG2 cells. Cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction and lactate dehydrogenase (LDH) release assays after 24 to 72 h of incubation. DNA damage was assessed by the comet assay, 24 h after incubation with the capped AuNPs. Uptake and subcellular distribution of the tested AuNPs was evaluated by quantifying the gold intracellular content by graphite furnace atomic absorption spectrometry (GFAAS) and transmission electron microscopy (TEM), respectively. The obtained results indicate that both differently coated AuNPs did not induce significant cytotoxicity. An inverse concentration-dependent increase in comet tail intensity and tail moment was observed in Cit-AuNPs- but not in MUA-AuNPs-exposed cells. Both AuNPs were internalized in a concentration-dependent manner. However, no differences were found in the extent of the internalization between the two types of NPs. Electron-dense deposits of agglomerates of Cit- and MUA-AuNPs were observed either inside endosomes or in the intercellular spaces. In spite of the absence of cytotoxicity, DNA damage was observed after exposure to the lower concentrations of Cit- but not to MUA-AuNPs. Thus, our data supports the importance of the surface properties to increase the biocompatibility and safety of AuNPs. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: gold nanoparticles; surface properties; cytotoxicity; DNA damage; cellular uptake; HepG2 cells

Introduction

As a result of their chemical, optical and magnetic properties, gold nanoparticles (AuNPs) are expected to be successfully applied in biomedicine for diagnostics (Baptista *et al*., 2011), drug/gene delivery (Petkar *et al*., 2011) and thermal tumour ablation (Cherukuri *et al*., 2010). Gold has traditionally been considered inert and biocompatible. However, higher reactivity than that observed in the bulk material can arise at a nanoscale, and consequently the assessment of the health risks associated with this class of NPs has become a critical issue.

The change of the surface chemical properties by exchanging the capping agent on the surface of the NPs is of great interest, as the capping can improve their stability and biocompatibility. It could also be useful to increase NPs plasma half-life by evading reticuloendothelial system (RES)-mediated capture and removal from systemic circulation (Niidome *et al*., 2006) or to provide specificity of the interaction with intended targets (Kumar *et al*., 2012). Surface modification using the known high affinity groups such as thiol (-SH), nitriles (-CN) and amine (-NH2) to gold surfaces has been proven to be very useful in changing the surface properties of AuNPs (Shimmin *et al*., 2004; Templeton *et al*., 2000).

At present, the knowledge of the interaction of nanoparticles with cells is reduced but it is known that the AuNPs, which bind easily to amine and thiol groups, can interfere with DNA (Gearheart *et al*., 2001) and amino acids (Selvakannan *et al*., 2004). In spite of the increasing number of studies addressing the potential toxicological effects of AuNPs, data are so far inconclusive (Alkilany and Murphy, 2010). Factors such as shape, size and surface chemistry are known to affect the AuNPs physicochemical properties, their interactions with biological systems, ultimately influencing their uptake and toxicity (Alkilany and Murphy, 2010; Cherukuri *et al*., 2010; Khlebtsov and Dykman, 2011). The AuNPs can produce adverse effects by interacting with vital cellular components, such as the plasma

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membrane, mitochondria or the nucleus (Unfried *et al.*, 2007). Among the described cytotoxic effects induced by the AuNPs are DNA damage, mutagenesis, oxidative stress, apoptosis and necrosis (Gao *et al.*, 2011; Li *et al.*, 2011; Pan *et al.*, 2009; Tedesco *et al.*, 2010; Thakor *et al.*, 2011; Vecchio *et al.*, 2012a).

Most of the available biodistribution studies have shown that the liver is a preferential target organ for AuNPs (Alkilany and Murphy, 2010; Khlebtsov and Dykman, 2011). Recently, an *in vivo* study conducted by our group showed that citrate- (Cit), 11-mercaptoundecanoic acid (MUA)- and pentapeptide-coated AuNPs (20 nm) preferentially accumulated in the rat liver after intravenous (i.v.) injection (Morais *et al.*, 2012). Therefore, the aim of the present study was to comparatively evaluate the role of the surface coating in the uptake, cytotoxicity and genotoxicity induced by 20 nm AuNPs in HepG2 cells, an *in vitro* model of human hepatocyte cells. Two differently capped AuNPs were employed: Cit- and MUA-coated AuNPs. These capping agents were chosen as they are commonly employed in the synthesis of AuNPs. Citrate acts as both a reducing and capping agent in AuNPs synthesis by the Turkevich method (Enustun and Turkevich, 1963; Kimling *et al.*, 2006) and the alkanethiol 11-MUA has proved effective as a surfactant allowing control of nanoparticle size but also facilitating surface functionalization (Lai *et al.*, 2011, 2012). The use of Cit- (Stobiecka *et al.*, 2010) and MUA-coated AuNPs (Lai *et al.*, 2012) is being investigated for biomedical purposes. Thus, cytotoxicity tests were conducted to evaluate whether the two capped AuNPs might affect cell metabolic activity or plasma membrane integrity. The DNA-damaging ability of the coated AuNPs was also examined using the comet assay. Uptake and subcellular localization of the tested AuNPs was determined by atomic absorption spectroscopy and transmission electron microscopy (TEM), respectively.

Material and Methods

Reagents

All chemicals used were of high purity or analytical grade. Nitric acid (HNO_3), hydrogen peroxide (H_2O_2), palladium nitrate [$Pd(NO_3)_2$], magnesium nitrate [$Mg(NO_3)_2$], hydrochloric acid (HCl), Triton X-100 and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). A gold pure calibration standard was obtained from Perkin-Elmer (Waltham, MA, USA). Tetrachloroauric acid ($HAuCl_4$; ~30 % wt), sodium citrate (Na_3Cit), 11-mercaptoundecanoic acid (11-MUA), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and methyl methanesulfonate (MMS) were purchased from Sigma-Aldrich (Madrid, Spain). Cell culture reagents were purchased from Gibco (Madrid, Spain).

Synthesis and characterization of the AuNPs

AuNPs were synthesized under strict sterile conditions using the Turkevich citrate reduction method with slight modifications (Enustun and Turkevich, 1963; Kimling *et al.*, 2006). Exchange of the citrate by the 11-mercaptoundecanoic acid was performed as previously described (Morais *et al.*, 2012), using a molar ratio capping agent/[AuNP] of 120. The concentration of the colloidal suspensions was determined by UV/vis spectroscopy using the method of Haiss *et al.* (Haiss *et al.*, 2007) and further confirmed by GFAAS. The average particle size of the initial batch (Cit-AuNPs) was determined by TEM using a HITACHI H-8100 microscope operated at 200 kV. The hydrodynamic diameter and zeta potential of the AuNPs in aqueous solution was measured by Dynamic Light Scattering (DLS) using a Zeta Sizer Nano ZS (Malvern Instruments, Worcestershire, UK), with a laser 4 mWHe-Ne (633 nm), at 25 °C.

Cell culture

HepG2 cells were kindly provided by Dr Maryam Modarai (Centre for Toxicology, School of Pharmacy, University of London, UK). The cells were cultured in Alpha Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin solution, 1% Fungizone and 6 $\mu g\,ml^{-1}$ transferrin. Cells were maintained in a 5% CO_2–95% air atmosphere at 37 °C and the medium was replaced every 2 days. Cultures were passaged at approximately 70% confluence using a 0.05% trypsin/0.53 mM EDTA solution to a maximum of 10 passages.

Handling and preparation of the AuNPs solutions

All procedures of handling and preparation of the AuNPs were standardized to minimize within-experiment variations. The experiments were performed using the same batch of AuNPs. The AuNPs stock suspensions were kept at room temperature, protected from light and remained stable without any detectable sign of precipitation or change of colour throughout the study. Different concentrations of AuNPs were freshly prepared from the stock solutions by direct dilution in complete cell culture medium. To ensure complete dispersion, the colloidal suspensions were sonicated for 5 min in an ultrasound bath (Bandelin Sonorex RK 100H; Berlin, Germany) before dilution in the culture medium.

Cytotoxicity assays

To examine the cytotoxicity induced by AuNPs, changes in the viability of HepG2 cells after incubation with the capped AuNPs were evaluated at confluence by assessing mitochondrial activity and membrane integrity by the MTT reduction and LDH release assays, respectively. Cells were seeded into 96-well plates (Falcon; BD Biosciences, Oxford, UK) at a density of 150 000 cells per well. After reaching confluence, culture media was gently aspirated and the cells were exposed to either the appropriate vehicle or to increasing concentrations of the coated AuNPs (0–200 μM Au) for 24, 48 and 72 h. The tested concentrations were within the range of those found in the literature (Alkilany and Murphy, 2010) and were selected based on the amount of Au present in the rat liver at 24 h after a single i.v. administration of the tested citrate- and MUA-capped AuNPs (Morais *et al.*, 2012).

After incubation with the AuNPs, plates were observed under a light microscope (Olympus CK 40) to detect NPs aggregates and to monitor morphological changes. At each selected time point, the incubation medium was removed and the cells were washed with Hanks balanced salt solution (HBSS) pH 7.4. For the MTT reduction assay, cells were incubated for 30 min with a solution of 0.25 $mg\,ml^{-1}$ MTT at 37 °C in a humidified, 5% CO_2-95% air atmosphere. Subsequently, the MTT solution was aspirated and the formed formazan crystals were dissolved in DMSO. The spectrophotometric analysis was run at 570 nm using a multi-well plate reader (PowerWave X; Bio-Tek, Winooski, VT, USA) with background subtraction at 690 nm. Cell viability

was calculated as the percentage of the viable cells compared with untreated controls. Cell death was determined by quantification of LDH release to the culture media, according the rate of oxidation of NADH at 36.5 °C measured at 340 nm, as previously described (Bergermeyer and Bernt, 1974). All viability experiments were conducted in at least three independent times, each run in triplicate. Results are presented as percentage relatively to the untreated condition.

Genotoxicity assay

Genetic instability is often associated with cancer development and emerging evidence has shown that nanomaterials can induce DNA damage (Singh *et al*., 2009). Therefore, the commonly used single cell gel electrophoresis assay (comet assay) was performed to assess the ability of AuNPs to cause DNA damage in HepG2 cells. Cells were seeded into 24-well plates at a density of 25 000 cells per well. Semiconfluent cultures were exposed for 24 h to four concentrations of either Cit- or MUA-AuNPs: 0.1, 1, 10 and 100 μM. Untreated controls and a positive control (cells treated with 50 μM MMS) were included in all series. The comet assay was performed in alkaline conditions (pH > 13) as described previously by Singh *et al*. (1988) with minor modifications described by Costa *et al*. (2008). All the steps described were conducted under a reduced light level to prevent additional DNA damage. Briefly, after the exposure period, media were aspirated and cells washed with phosphate-buffered saline (PBS) pH 7.4. Next, the cells were gently harvested to a tube and centrifuged at 300 *g* for 10 min. The pellet was resuspended in PBS pH 7.4 and cell suspensions were tested for viability by the trypan blue exclusion, which was consistently found to be > 90%. Aliquots of 1×10^5 cells were centrifuged at 400 *g* for 5 min. The pellets were resuspended in 0.5% low-melting-point agarose and layered onto dry microscope slides (VWR, Darmstadt, Germany) pre-coated with 1% normal-melting agarose. The agar was allowed to solidify on ice for 10 min, and thereafter the slides were immersed in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris Base, 2% Triton X-100) for 2 h at 4 °C, after which they were transferred to an electrophoresis tank containing freshly made electrophoresis buffer (1 mM EDTA, 300 mM NaOH pH > 13), where they were kept for 40 min at 4 °C to allow DNA unwinding. Electrophoresis was performed in the same buffer for 20 min at 30 V and 300 mA. The slides were then neutralized with 0.4 M Tris base pH 7.5, air-dried and the DNA was stained with a 20 μg ml^{-1} ethidium bromide solution. The slides were coded, and one scorer performed the comet analysis using a fluorescence microscope and the image analysis software Comet Assay IV (Perceptive Instruments, Suffolk, UK). The percentage of DNA in the comet tail and the olive tail moment were used as a measure of the amount of DNA damage. A hundred cells per slide were counted and three independent experiments were performed in triplicate.

Cellular uptake of AuNPs

Surface chemistry influences the degree of internalization and the magnitude of the toxicity effects induced by NPs has been attributed to their ability to penetrate cells (Alkilany and Murphy, 2010). Thus, to evaluate the influence of the capping agents in the cellular uptake of the AuNPs, HepG2 cells were grown to confluence and incubated for 24 h to four different concentrations of Cit- and MUA-coated AuNPs (10, 30, 100 and 200 μM) at 37 °C in a humidified 5% CO_2/95% air atmosphere. The experiment was terminated by aspirating the NPs solution and washing the cell monolayers with HBSS pH 7.4 to remove unspecific binding of AuNPs. Cells were lysed using a 0.05% trypsin/0.53 mM EDTA solution. Complete cell medium was added to inactivate the trypsin. The resulting cell suspensions were digested using aqua regia. After overnight digestion at 105 °C, the samples were allowed to cool at room temperature and 37% HCl was added. Then, the samples were placed again in the oven at 105 °C for 24 h. At the end of the incubation time, samples were transferred into a tube and stored in the dark at room temperature, prior to the Au quantification. Elemental gold content of the acid-digested samples was determined by GFAAS using an AAnalyst 600 Atomic Absorption Spectrophotometer (Perkin-Elmer) equipped with an autosampler as previously described (Morais *et al*., 2012). Protein content of the cell suspensions was determined by the Lowry method using the Bio-Rad DC protein assay. Results were expressed in μg of Au per mg of protein. The uptake experiments were conducted at least three independent times, each performed in triplicate.

Subcellular localization of AuNPs by TEM

Subcellular localization has also been reported to be influenced by the physicochemical properties of the AuNPs (Alkilany and Murphy, 2010). Differential distribution of AuNPs will dictate interactions with different cellular components. Therefore, information on NPs location provides important clues about nanomaterials-induced effects and associated mechanisms. Thus, TEM analysis was performed to further confirm the uptake and observe the subcellular localization of the capped AuNPs in HepG2 cells. For that purpose, cells were grown on 24-well plates and exposed for 24 h to the AuNPs (0.1, 10 and 100 μM) at 37 °C in a humidified 5% CO_2/95% air atmosphere. At the end of the incubation period, monolayers were washed with HBSS pH 7.4 to remove unbound NPs, the cells were gently detached with a 0.05% trypsin/0.53 mM EDTA solution and collected to a tube containing complete cell medium to inactivate the trypsin. Cell suspensions were centrifuged at 300 g, for 10 min, and the pellet fixed with 2.5% glutaraldehyde in 0.2 M sodium cacodylate pH 7.2–7.4 for 2 h, post-fixed with 2% osmium tetroxide, dehydrated through graded alcohol solutions and embedded in Epon. Ultrathin sections were mounted on copper grids and contrasted with uranyl acetate and lead citrate for TEM analysis (Zeiss EM 10A; Carl Zeiss, Oberkochen, Germany).

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Data analyzes were performed using the GraphPad Prism 5 software (San Diego, CA, USA). Parametric analyzes were performed using the one-way ANOVA with post-hoc Bonferroni's tests applied for multiple comparisons. Non-parametric analysis and comparisons between groups were performed with the Kruskal–Wallis test followed by Dunn's test. Nanoparticle uptake data was analyzed by simple linear regression. Significance was accepted at a *P*-value ≤0.05.

Results

Synthesis and characterization of AuNPs

The main characteristics of the negatively charged AuNPs used in our study are summarized in Table 1 and Fig. 1. Spherical

Table 1. Main characteristics of the gold nanoparticles (AuNPs) used in the study. The average size and zeta potential of the AuNPs was determined by Dynamic Light Scattering (DLS). The Au content of the suspensions was determined by Graphite Furnace Atomic Absorption Spectrometry (GFAAS)

	Citrate-coated AuNPs	MUA-coated AuNPs
Hydrodynamic radius (nm)[a]	22.4 (5.7)	33.2 (9.8)
Zeta Potential (mV)[b]	- 44.7 ± 7.5	- 37.3 ± 8.4
[NP] (nM)[c]	4.67	4.44
[Au] ($\mu g\,ml^{-1}$)[d]	249.0 ± 35.3	236.5 ± 15.4

[a]The values in parenthesis are polydispersion indexes.
[b]Results are expressed as mean ± standard deviation.
[c]The concentration of NPs was evaluated by UV/vis.
[d]The concentration of Au of the stock colloidal suspensions was determined by GFAAS. Results are expressed as mean ± standard error of the mean.

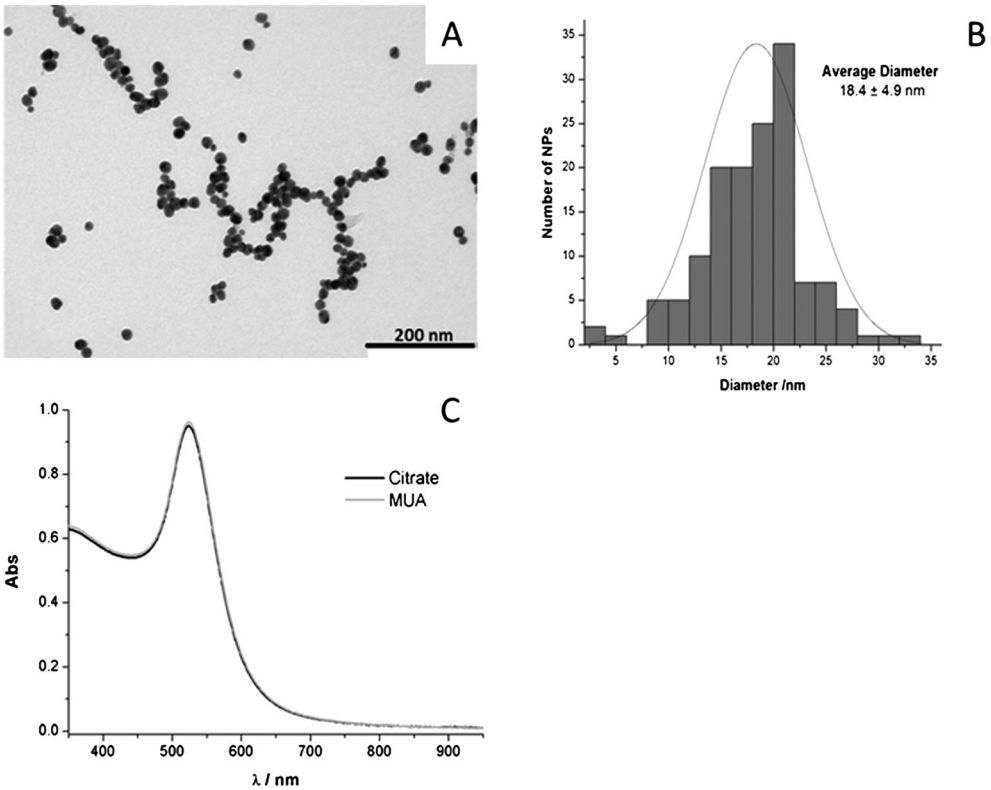

Figure 1. (A) Representative transmission electron microscopy (TEM) image of citrate-gold nanoparticles (AuNPs). The bar represents 200 nm; (B) histogram of size distribution of citrate-AuNPs with an average diameter of 18 ± 5 nm; (N = 143 particles); (C) UV-vis spectra of colloidal solutions of AuNPs capped with citrate and 11-mercaptoundecanoic acid (MUA).

citrate-stabilized AuNPs (Cit-AuNPs) were prepared as described by Turkevich *et al.*, with minor modifications (Enustun and Turkevich, 1963; Kimling *et al.*, 2006) in strict aseptic conditions. MUA-stabilized AuNPs (MUA-AuNPs) were obtained by addition of MUA to Cit-AuNPs (120:1 molar ratio), and overnight incubation. Excess of MUA and citrate was removed by centrifugation and redispersion of the nanoparticles in water. All of the experimental procedure was performed in strictly aseptic conditions. Analysis of the UV-vis spectra of Cit- and MUA-coated AuNPs revealed the presence of a plasmon absorption band at 520 nm, a characteristic feature attributed to the formation of AuNPs (Fig. 1C). The average NP diameter measured by TEM (Cit-AuNP) was 18.4 ± 4.9 nm (N = 134 NPs). This agrees well with the hydrodynamic diameter in aqueous solution determined by DLS for both types of particles, shown in Table 1. The zeta potential of the colloidal dispersions of AuNPs was also determined and the results are shown in Table 1. Both NPs show a high negative surface charge owing to the negatively charged citrate or MUA. The negative charge provided by these anions imparts the colloidal stability in aqueous media necessary for the present experimental studies (Sperling and Parak, 2010). The concentration of Cit-AuNPs (Table 1) was evaluated by UV/vis spectroscopy using the method of Haiss *et al.* (2007). The gold content of the AuNPs suspensions [(Au) in $\mu g\,ml^{-1}$] was further confirmed by GFAAS (Table 1).

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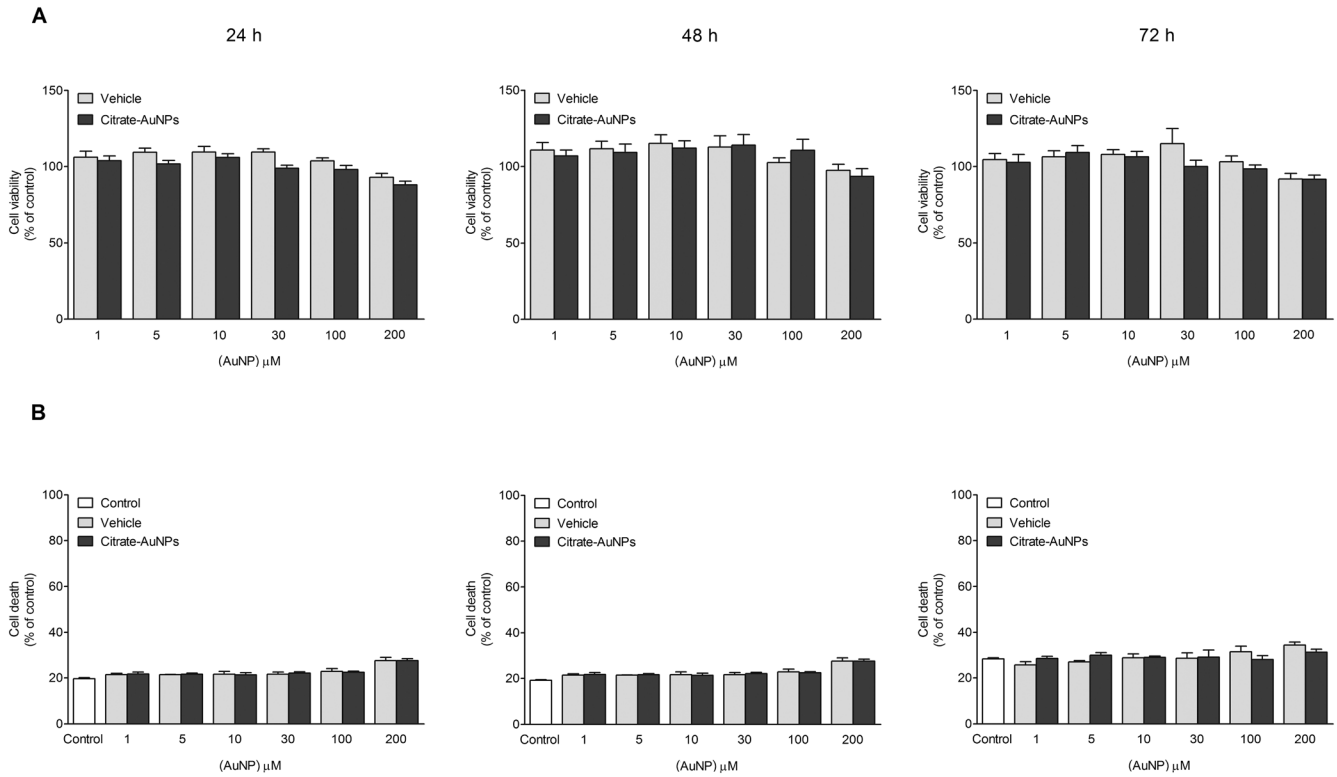

Figure 2. Effect of citrate-gold nanoparticles (AuNPs) on (A) cell viability assessed by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction and (B) lactate dehydrogenase (LDH) leakage in HepG2 cells. Cells were exposed to different concentrations of AuNPs (0–200 μM Au) for 24, 48 and 72 h. Results were calculated as percentage of control (untreated cells) and data are presented as mean ± standard error of the mean (SEM) ($n = 4$ per group).

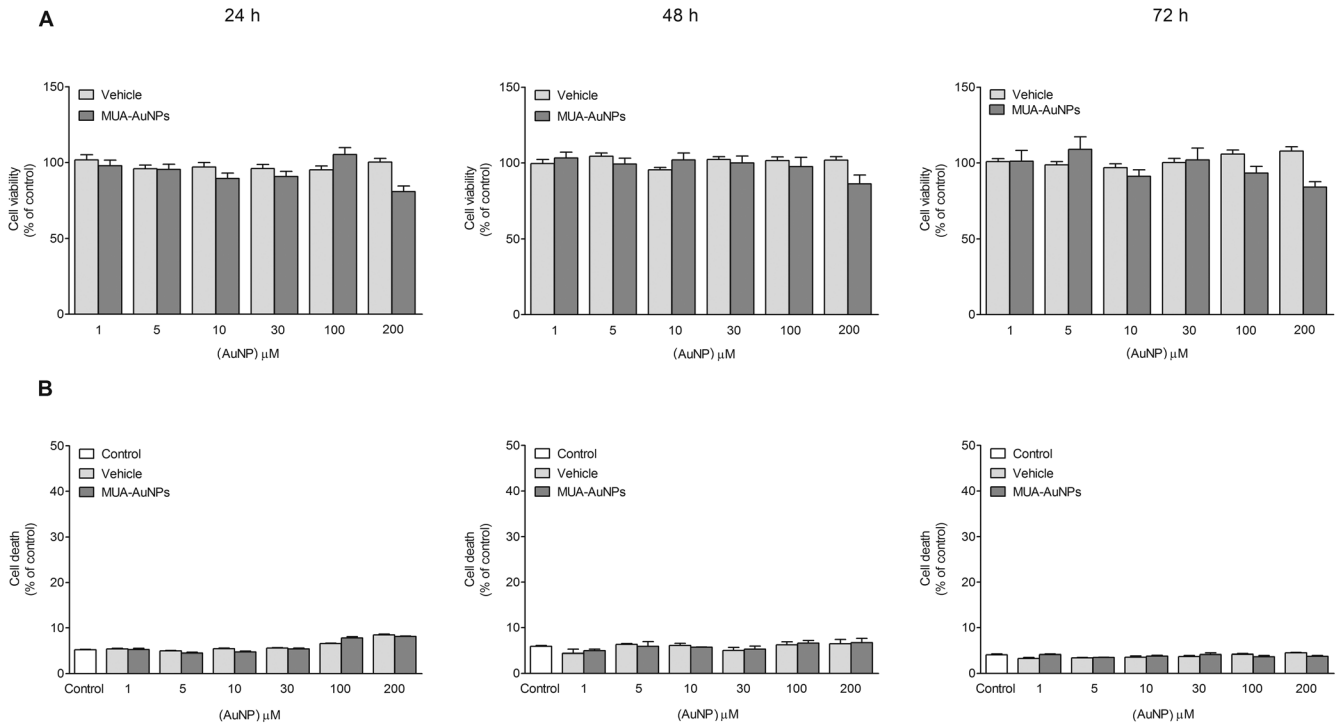

Figure 3. Effect of 11-mercaptoundecanoic acid (MUA)-gold nanoparticles (AuNPs) on (A) cell viability assessed by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction and (B) lactate dehydrogenase (LDH) leakage in HepG2 cells. Cells were exposed to different concentrations of the AuNPs (0–200 μM Au) for 24, 48 and 72 h. Results were calculated as percentage of control (untreated cells) and data are presented as mean ± standard error of the mean (SEM) ($n = 4$ per group).

Cytotoxicity assays

Figures 2 and 3 show the results of the assessed toxicity endpoints, mitochondrial activity and plasma membrane integrity, respectively. As shown in Fig. 2, no changes in cell mitochondrial activity, as determined by the MTT reduction assay, were observed after exposure to the vehicles, Cit-AuNPs or MUA-AuNPs up to 200 μM. Under the same experimental conditions, the LDH assay indicated that the plasma membrane integrity of cells incubated with Cit- or MUA-AuNPs was not compromised (Fig. 3). Thus, these results confirm that no cytotoxicity was observed after exposure to the vehicles, Cit-AuNPs or MUA-AuNPs, even after 72 h of continuous incubation with the AuNPs as compared with untreated cells.

Genotoxicity assay

As represented in Table 2, exposure of HepG2 cells for 24 h to Cit-AuNPs but not to MUA-AuNPs induced significant damage as compared with untreated cells. Interestingly, this damaging effect was inversely proportional to the concentration, i.e. lower concentrations (0.1, 1 and 10 μM) were more effective in inducing damage than higher ones. Vehicle-treated cells did not exhibit any signs of DNA damage. Images of the comet assay of control, vehicle- and low-concentration Cit-AuNPs-treated HepG2 cells are represented in Fig. 4. As shown, control (A) and vehicle-treated cells (B) show residual or no tail, whereas cells exposed to 0.1 μM Cit-AuNPs exhibit a significantly higher damage compared with untreated cells, as confirmed by the % of tail intensity (34.43 ± 4.08 vs. 14.45 ± 1.55) and the olive tail moment (6.28 ± 1.18 vs. 1.81 ± 0.23) values (Table 2). The higher concentration tested of Cit-AuNPs (100 μM) failed to induce any significant damage. This behavior may be related to higher aggregation of Cit-AuNPs at higher concentrations, but further studies are necessary to confirm this hypothesis.

Table 2. Comet assay analysis of DNA damage in HepG2 cells exposed to the gold nanoparticles (AuNPs)

	Tail intensity (%)	Tail moment
Control	14.45 ± 1.55	1.81 ± 0.23
Vehicle	15.96 ± 2.93	2.44 ± 0.63
Citrate-AuNPs (μM)		
0.1	34.43 ± 4.08*	6.28 ± 1.18*
1	27.61 ± 3.02*	4.72 ± 0.79*
10	24.39 ± 3.02*	3.98 ± 0.65
100	22.68 ± 2.68	3.90 ± 0.63
MUA-AuNPs (μM)		
0.1	17.35 ± 4.87	2.43 ± 0.89
1	20.42 ± 2.78	3.43 ± 0.71
10	21.14 ± 2.78	3.42 ± 0.61
100	21.54 ± 2.96	3.36 ± 0.61
MMS 50 μM	55.88 ± 2.91**	13.94 ± 1.91**

Cells were treated with vehicle or AuNPs for 24 h. The alkylating agent methyl methanesulfonate (MMS) was used as a positive control. Data represents the mean ± standard error of the mean (SEM) of three independent experiments ($n = 3$ per group).

* $P < 0.05$, ** $P < 0.001$; significantly different from the control value.

Uptake and subcellular localization of the AuNPs

The cellular uptake of both Cit- and MUA-AuNPs was determined 24 h after cell incubation with increasing concentrations of the NPs, based on the intracellular Au content quantified by GFAAS. For the tested concentrations, the uptake of both Cit- and MUA-AuNPs by the HepG2 cells at 37 °C increased linearly ($R^2 > 0.98$) (Fig. 5) but no significant differences were observed in the rate of internalization between Cit- and MUA-AuNPs. These findings were further confirmed by qualitative TEM analysis of fixed HepG2 cells exposed for 24 h to 0.1, 10 or 100 μM of both types of AuNPs (Fig. 6). Electron microscopy revealed the presence of intracellular membrane-bound vesicles containing electron-dense deposits of NPs agglomerates in cells for all the tested concentrations of Cit-AuNPs. Electron-dense material was also detected in the intercellular spaces. Similar findings were observed in MUA-AuNPs-exposed cells but only for concentrations higher than 0.1 μM. A concentration-dependent increase in the volume of the NPs-containing vesicles was observed in cells treated with both types of AuNPs. No detectable signs of freely dispersed AuNPs in the cytoplasm, in the perinuclear region or in other cytoplasmic organelles were found. Signs of apoptosis and necrosis were observed in cells exposed to Cit- and MUA-AuNPs.

Discussion

In spite of the increasing number of previous studies regarding the safety assessment of the AuNPs, there is still some uncertainty about the toxicological potential of the AuNPs supported by the often conflicting literature data (Alkilany and Murphy, 2010). In this study, we evaluated the influence of the surface coating

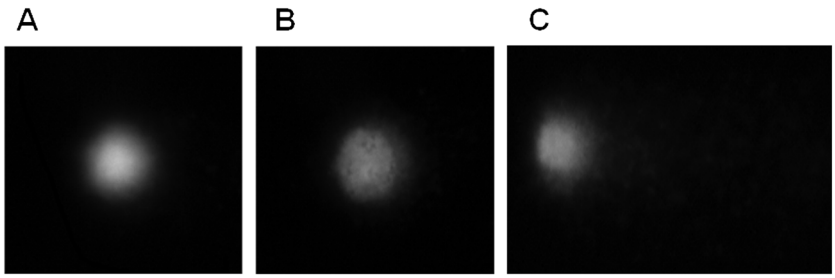

Figure 4. Representative images of the comet assay in HepG2 cells. Control (A) and vehicle (B) cells show residual to no tail, whereas 0.1 μM citrate-gold nanoparticles (AuNPs) exposed cells (C) comparatively display a longer tail, indicative of a presence of higher DNA damage.

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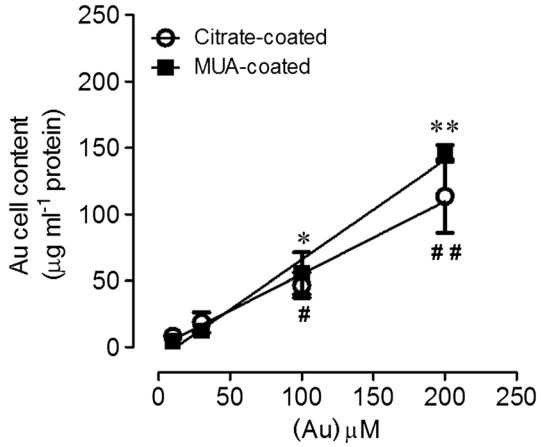

Figure 5. Uptake of the gold nanoparticles (AuNPs) in the HepG2 cells. Cells were exposed to different concentrations of the AuNPs (0–200 μM Au) for 24 h at 37 °C in a humidified 5% CO_2/95% air atmosphere. Intracellular Au content was determined by Graphite Furnace Atomic Absorption Spectrometry (GFAAS). Data are expressed as mean ± standard error of the mean (SEM) ($n = 4$ per group). $^{\#}P < 0.05$ $^{\#\#}P < 0.001$ vs. 10 μM citrate (Cit)-AuNPs; $^{*}P < 0.05$ $^{**}P < 0.001$ vs. 10 μM MUA-AuNPs. No significant differences were found between equal concentrations of Cit- and MUA-AuNPs.

in the uptake, cytotoxic and genotoxic effects induced by AuNPs on HepG2 cells, an *in vitro* model of hepatic cells. Under our experimental conditions, no changes in HepG2 mitochondrial activity or increased cell death were observed in response to exposure to either Cit- or MUA-coated AuNPs, as assessed by the MTT reduction and LDH release assays. Our results are in agreement with those reported by Singh *et al*. (2010) that have found that glycolipid capped-AuNPs did not induce cytotoxicity on HepG2 cells up to 100 μM of the Au concentration. However, Jan *et al*. (2008) have found that AuNPs inhibited the proliferation and intracellular calcium release of HepG2 cells. These findings suggest that surface chemistry plays an important role in the interactions between AuNPs and cells.

Surface modification with 11-MUA did not alter the uptake of the AuNPs by the HepG2 cells relative to Cit-AuNPs. Both differently coated AuNPs were efficiently taken up in a concentration-dependent manner by HepG2 cells but no differences were observed in the extent of the internalization between the two types of NPs. These results are in agreement with our previous *in vivo* experiments that have shown no significant differences in the hepatic accumulation levels of Au at 24 h after injection into the rat tail vein of Cit- or MUA-coated AuNPs (Morais *et al*., 2012).

Increasing evidence demonstrates that nanomaterials can induce genotoxic responses through a variety of mechanisms that may include activation of inflammatory or oxidative cascades (Singh *et al*., 2009). In the present study, the comet assay was selected to assess DNA damage as it has been the most often used test to assess the genotoxicity of nanomaterials (Landsiedel *et al*., 2009). Our results demonstrate that surface modification might be effective in protecting cells from the adverse effects of the AuNPs upon the DNA as data obtained showed that MUA-capped AuNPs did not induce any damage in the DNA, whereas a positive result was observed in Cit-AuNPs-exposed cells.

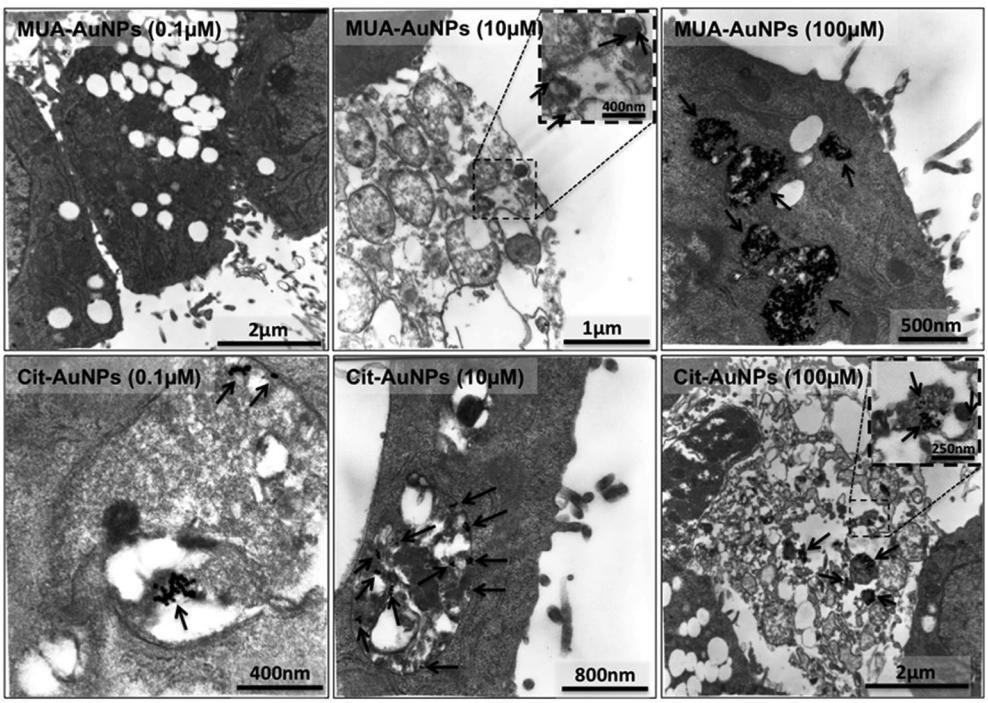

Figure 6. Transmission electron micrographs (TEMs) obtained from cultures of HepG2 cells incubated for 24 h with different concentrations (0.1, 10 and 100 μM) of gold nanoparticles (AuNPs) coated with 11-mercaptoundecanoid acid (MUA-AuNPs) or with citrate (Cit-AuNPs), showing the intracellular distribution of internalized AuNPs (arrows). In spite of the biochemical detection of gold, it was not possible to observe AuNPs within cells exposed to 0.1 μM of MUA-AuNPs. All the observed intracellular AuNPs were located inside endosomes (arrows). No signs of nanoparticles dispersed in the cytosol or within the nucleus have been detected. Micrographs of MUA-AuNPs (10 μM) and Cit-AuNPs (100 μM) depict necrotic cells with AuNPs inside endosomes, as shown by the magnification of the dashed square marked in both photos.

DNA damage after exposure to Cit-AuNPs has been previously reported. Vecchio *et al*. (2012a, 2012b) observed significant AuNPs-induced toxicity *in vivo*, namely a strong reduction of Drosophila lifespan and fertility performance, DNA fragmentation, as well as a significant modification in the expression levels of genes involved in stress responses, DNA damage recognition and the apoptosis pathway being the observed toxic effects directly proportional to the administered dose of Cit-AuNPs. Kang *et al*. (2010) also reported a substantial DNA damage and cell cycle arrest in human oral squamous cell carcinoma after exposure to AuNPs capped with specific peptides to selectively transporting them to the nuclei, demonstrating that AuNPs localized in the nuclei have important implications in cell function and survival.

At present, the relevance and validity of *in vitro* studies to evaluate nanomaterial genotoxic potential is being questioned. Although there is a well-defined battery of tests and guidelines to assess genotoxicity of novel chemicals, the choice of the relevant regulatory testing of nanomaterials is still being discussed (Doak *et al*., 2012). The genotoxicity testing of nanomaterials is rather complex owing to the variety of administration routes, doses and duration of exposure. *In vitro* genotoxicity studies constitute a valuable tool to get a better understanding of the complexity of the nanomaterial-biological interactions in a simple model, elucidating on the nature of the associated toxicity mechanisms, which can be useful to predict *in vivo* toxicity. However, *in vitro* testing has some limitations in mimicking *in vivo* conditions, which do not invalidate its use as long as data correlate well with *in vivo* findings.

Owing to the inert nature of the bulk gold, *in vivo* studies on the genotoxic potential of AuNPs are still scarce and the available studies on the DNA-damaging ability of the AuNPs are so far inconclusive. Girgis *et al*. (2012) have studied the genotoxic effects of 15 nm Au NPs and gold-cobalt nanoparticles (Au-Co NPs) at 7 and 14 days after oral administration in mice. These authors have found that with the exception of the highest tested dose, AuNPs had lower effects than those of Au-Co NPs on alteration in the tumor-initiating genes, frequency of MNs and generation of 8-OHdG as well as glutathione peroxidase activity. These results suggested that the potential to cause *in vivo* genetic and antioxidant enzyme alterations may be attributed to the increase in oxidative stress in mice. On the other hand, no DNA damage in the lung tissue as assessed by the comet assay or micronucleation in polychromatic erythrocytes of the bone marrow, were observed by Schulz *et al*. (2012) at 72 h after a single instillation of differently sized (2, 20 and 200 nm) AuNPs into the trachea of male adult Wistar rats. *In vivo* genotoxicity assessment with our test AuNPs is already planned and will hopefully provide valuable information about the relevance of the present *in vitro* findings.

Most of the available nanotoxicological studies focus on the cytotoxicity induced by high concentrations of nanomaterials. However, effects that arise at lower concentrations had been neglected. As a result of their high surface area/volume ratio nanoparticles exhibit a greater reactivity compared with bulk material. This characteristic might account for the fact that most of the available studies on the effects induced by the nanoparticles found a binary 'all-or-nothing' phenomenon rather than a gradual, concentration-dependent process. In our study, the genotoxic effects were inversely proportional to the tested concentrations of Cit-AuNPs. This unusual inverse relationship between toxic effects and concentration has been recently acknowledged for NPs, demonstrating the complexity of their hazard. Sergent *et al*. (2012) have found that low concentrations of 100 nm SiO_2-NPs induced higher cytotoxic/genotoxic effects on human colonic HT-29 cells than high concentrations after 24 h of exposure. Similar findings have also been observed *in vivo*. In a study aimed at evaluating the toxicological potential of zinc oxide nanoparticles, Pasupuleti *et al*. (2011) found an inverse dose-dependent increase in aspartate aminotransferase and alanine aminotransferase serum levels at 14 days after an acute oral administration of zinc oxide nanoparticles to rats. Also, the incidences of microscopic lesions in the liver, pancreas, heart and stomach were higher in the groups treated with lower doses compared with the group treated with the higher dose of zinc oxide nanoparticles (Pasupuleti *et al*., 2011). Regarding the AuNPs tested in the present study, it is expected that in biological media citrate capping should be easily replaced by other molecules such as proteins mainly through interactions with cysteine and histidine residues. However, this coating replacement is not likely to occur in MUA-AuNPs owing to the high-affinity bond between gold and the thiol group of MUA. Thus, Cit-AuNPs are more prone to aggregation in biological media than MUA-coated AuNPs. Therefore, we first hypothesized that high concentrations of Cit-AuNPs resulted in more aggregation and low ability of the NPs to reach the perinuclear region and induce direct DNA damage. However, TEM findings failed to support this hypothesis. In fact, Cit-AuNPs, even at low concentrations, were consistently found inside membrane-bound vesicles containing electron-dense deposits of NPs agglomerates and no sign of free NPs in the cytosolic or perinuclear region was detected. HepG2 cells have been previously reported to possess DNA repair systems (Benhusein *et al*., 2010), which can be involved in the defense against the genotoxic effects of AuNPs. Protective mechanism(s) activated in response to high levels of AuNPs could thus be responsible for this inverse correlation.

In conclusion, our study compared the effects induced on HepG2 cells by the two types of AuNPs: citrate-coated compared with MUA-coated AuNPs. Regardless of the different surface coating, no differences were observed in the levels of uptake and toxicity as evaluated by the MTT and LDH release assays. Internalization of the AuNPs was confirmed by TEM and signs of apoptosis and necrosis were observed. Exposure for 24 h to low concentrations of Cit-AuNPs produced DNA damage in HepG2 cells. Further studies should be carried out to better investigate the mechanism involved and to further characterize the nature of DNA damage. Our data underline the importance of assessing different toxicity endpoints to get a better understanding of the global cellular effects and mechanisms associated with AuNPs. It is also pointed out that AuNPs, even at low concentrations, can produce DNA lesions. This is an important finding that should raise awareness to the occupational and health risks that might be associated with exposure to low levels of nanoparticles, in this particular case DNA damage, which might lead to genetic instability, a cancer risk factor. DNA damage was observed in Cit-AuNPs-exposed cells but not in MUA-AuNPs-exposed cells, supporting that surface modification can be a good strategy to protect cells from the undesirable effects of AuNPs on DNA.

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