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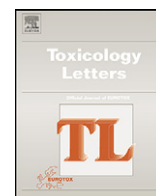Artigos e Materiais de Revistas Científicas - IFSC/FCI

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Cyto and genotoxicity of gold nanoparticles in human hepatocellular carcinoma and peripheral blood mononuclear cells

Cyto and genotoxicity of gold nanoparticles in human hepatocellular carcinoma and peripheral blood mononuclear cells

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HIGHLIGHTS

► The geno- and cytotoxicity of gold nanoparticles in cancer and healthy cells were investigated.
► The reactive oxygen species (ROS) generation was evidenced for both cells.
► The healthy cells exhibit less sensitive to DNA damage than cancer cells.
► The nanoparticulate systems can be applied in cancer therapy with reduced side effects.

GRAPHICAL ABSTRACT
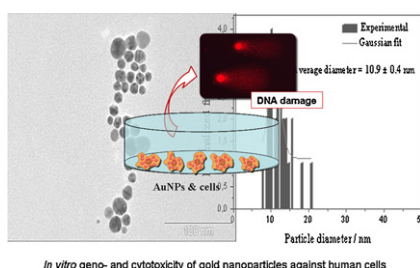
In vitro geno- and cytotoxicity of gold nanoparticles against human cells

ARTICLE INFO

ABSTRACT

Engineered nanomaterials have been extensively applied as active materials for technological applications. Since the impact of these nanomaterials on health and environment remains undefined, research on their possible toxic effects has attracted considerable attention. It is known that in humans, for example, the primary site of gold nanoparticles (AuNps) accumulation is the liver. The latter has motivated research regarding the use of AuNps for cancer therapy, since specific organs can be target upon appropriate functionalization of specific nanoparticles. In this study, we investigate the geno and cytotoxicity of two types of AuNps against human hepatocellular carcinoma cells (HepG2) and peripheral blood mononuclear cells (PBMC) from healthy human volunteers. The cells were incubated in the presence of different concentrations of AuNps capped with either sodium citrate or polyamidoamine dendrimers (PAMAM). Our results suggest that both types of AuNps interact with HepG2 cells and PBMC and may exhibit in vitro geno and cytotoxicity even at very low concentrations. In addition, the PBMC were less sensitive to DNA damage toxicity effects than cancer HepG2 cells upon exposure to AuNps.

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1. Introduction

Metallic nanoparticles have attracted enormous scientific and technological interest, mainly due to their unique, size dependent properties that allow their use as active materials in food, cosmetic, clothing, and biomedical areas (Kreuter and Gelperina, 2008; Johnston et al., 2010). Gold nanoparticles (AuNps), in particular, have been extensively designed and applied in biomedicine, especially for drug delivery, molecular imaging and cancer therapy (Alkilany and Murphy, 2010; Lewinski et al., 2008). As example, a new chemotherapy strategy has been proposed by Tomuleasa et al. (2012) regarding the use of AuNps conjugated with conventional chemotherapy drugs. The authors observed that the proliferation of hepatocellular carcinoma cancer cells was lower for cultures exposed to AuNps/chemotherapy drugs conjugates, in comparison

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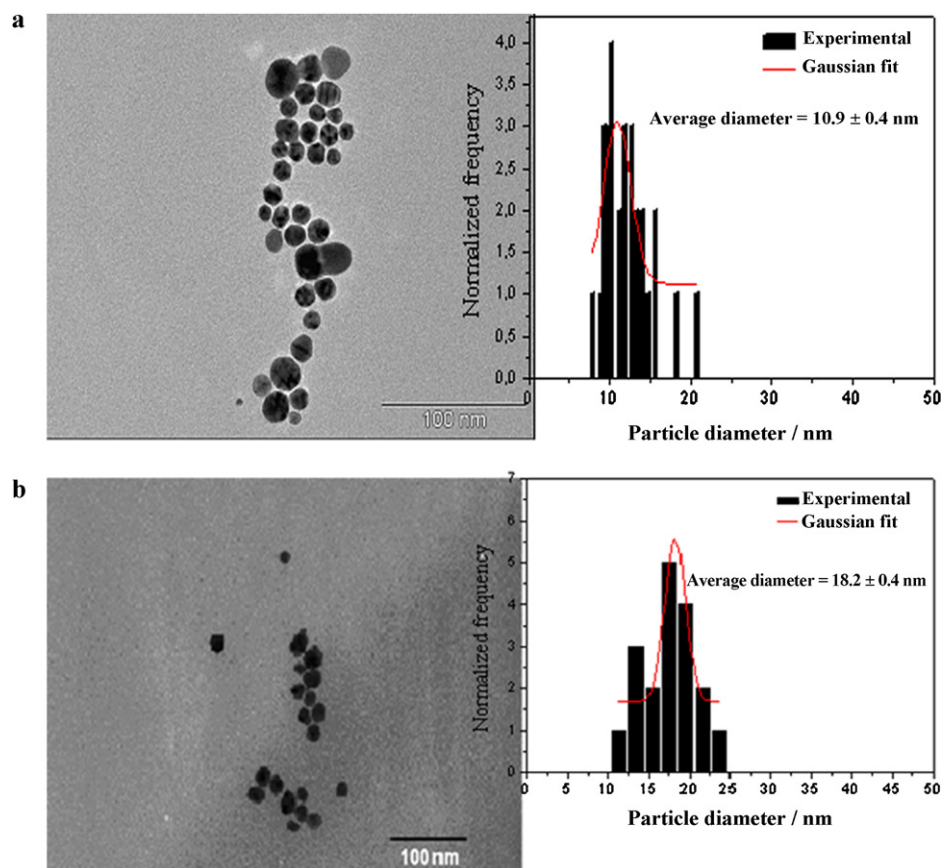E-mail address: iedammp@hotmail.com (I.M.M. Paino).

Fig. 1. TEM images and size distribution revealing the size and shape of a cluster of PAMAM (a) and citrate coated AuNps (b).

to cultures exposed to isolated cytostatic drugs (Tomuleasa et al., 2012).

Due to their possible use in biomedical areas, AuNps have been subject of research regarding the potential risks related to human exposure, upon investigating their interaction with biomolecules, cells and tissues (Maurer-Jones et al., 2009). Small AuNPs (1 nm in diameter) can easily cross the cell membrane and nucleus, and attach to the DNA (Tsoli et al., 2005). A study by Thakor et al. (2011) showed that the treatment with AuNps in HeLa or HepG2 cell lines caused no cytotoxicity at lower concentrations; however, cytotoxicity was observed at higher AuNps concentrations, after prolonged, continuous exposure to AuNps, in both cell lines. Pernodet et al. (2006) also demonstrated that citrate-coated AuNps affected human dermal fibroblast cell lines. On the other hand, a study by Connor et al. (2005) reported that 18 nm-diameter AuNps exhibited significant penetration into cells, but surprisingly, cytotoxicity was not observed.

The human hepatoma (HepG2) cells have been chosen as experimental models for *in vitro* toxicological studies (Wei et al., 2007) mainly because *in vivo* studies have demonstrated that the primary site of AuNps accumulation is the liver (Ogawara et al., 1999; Schipper et al., 2009; Sonavane et al., 2008). The latter has been also reported by Johnston et al. (2010), who demonstrated that the uptake of 20 nm polystyrene nanoparticles by primary rat hepatocytes and human hepatocyte cell lines (C3A and HepG2) was size and time dependent.

Several studies have also investigated the genotoxic potential of nanoparticles upon examining the extent of DNA damage using the comet assay (Collins, 2004). This methodology has become one of the standard methods for assessing DNA damage, with applications in nanotoxicology. The alkaline version of comet assay is one of the most important tools in a wide variety of cell lines for evaluation of

genotoxicity or DNA damage, upon calculating the DNA migration (Piperakis, 2009).

Since scarce studies have reported the effects of AuNps on HepG2 cells, especially on human cells from the immune system, the objective of this paper is to investigate the cyto and genotoxicity of AuNps stabilized with polyamidoamine (PAMAM) dendrimer and sodium citrate against HepG2 cells and Peripheral Blood Mononuclear Cells (PBMC) from healthy human volunteers. Our findings may reduce the serious lack of information and controversial studies concerning the toxicological effects of engineering gold nanoparticles.

2. Materials and methods

2.1. Materials synthesis and characterization of AuNps

Chemicals were obtained from Sigma–Aldrich (USA). Glutamine, penicillin/streptomycin, fetal bovine serum, cell culture media were purchased from Cultilab (Brazil). Doxorubicin (DXR) was used in commercial formula: Adriblastin*1* RD (CAS: 25316-40-9, Pharmacia and Upjohn, Milan, Italy).

AuNps were chemically synthesized in the presence of PAMAM or sodium citrate, leading to the formation of AuNps with diameters ranging from 7 to 20 nm, bearing positive and negative charges, respectively. Details on the synthesis of the Nps-PAMAM can be found elsewhere (Crespilho et al., 2007). Briefly, 2 mL of PAMAM G4 (0.07 mmol L^{-1}) were added to 2 mL of $HAuCl_4$ solution (1 mmol L^{-1}) and 2 mL of formic acid 10% (v/v). This solution was mixed and shaked during 4 h. The color changed from yellow to red, indicating the zerovalent Au complex was formed after 4 h.

The AuNps-citrate were obtained by citrate reduction of gold salts, as previously described (Grabar et al., 1995). Briefly, 1.0 mL of 1% sodium citrate was added to 14 mL of boiling solution 0.5 mmol L^{-1} $HAuCl_4$ with vigorous stirring. The final solution color changes to red–violet rapidly. The nanoparticle formation was monitored by UV–vis spectrophotometry (Hitachi U-2001 Spectrophotometer; San Jose, CA, USA). AuNPs morphology and particle size distribution were estimated by transmission electron microscopy (TEM, Model CM200; Philips, the Netherlands) by measuring at least 100 particles in TEM images using the program Image J

Table 1

Zeta potential and hydrodynamic diameter values of PAMAM and citrate-covered AuNps before and after dilution in cell culture medium supplemented with 10% FBS.

	AuNp-citrate	AuNp-PAMAM
Zeta potential (mV) as prepared	−15.0 ± 3.0	+41.0 ± 3.0
Zeta potential (mV) in medium cell culture containing 10% FBS	−1.2 ± 0.3	−6.8 ± 0.5
Hydrodynamic diameter (nm) as prepared	7.3 ± 1.2	7.2 ± 2.7
Hydrodynamic diameter (nm) in medium cell culture containing 10% FBS	10.1 ± 0.3	12.1 ± 3.3

Data are reported as means ± standard deviation (SD) of triplicate experiments.

(Java-Sun Microsystems). Typical AuNPs TEM images used in this study are shown in Fig. 1. The Zeta potential and the hydrodynamic diameter were measured (Malvern Zetasizer) before and after AuNp dilution into cell culture medium with serum (10% fetal bovine serum-FBS) (Table 1).

The citrate or PAMAM excess was removed upon successive centrifugation and rinsing steps using phosphate buffer saline 0.05 M (PBS) solution. After each centrifugation, the AuNps were resuspended in 0.05 M PBS at pH 7.0 following the discard of the supernatant. The process was repeated three times to eliminate the free citrate or PAMAM molecules. The AuNps were then diluted in cell culture medium. Finally, the AuNps were sonicated for 30 min before using.

2.2. Isolation of human peripheral blood mononuclear cell (PBMC)

Whole peripheral blood was collected from women and men adult healthy volunteers, no tobacco users, no pregnant women. They were informed about the experimental procedures, signed consent form to participate in the study, and all the procedures were approved by the Ethics Committee of the Federal University of São Carlos in keeping with the principles enunciated in the Declaration of Helsinki. All subjects were not taking any type of medication. The PBMC isolation was made by the difference of gradient density Ficoll-Hypaque (Histopaque®, Sigma–Aldrich-USA) 1077. After centrifugation ($400 \times g$; 30 min at room temperature), the PBMC were found at the plasma/1077 interphase and collected carefully with a Pasteur pipette. After that, the cells were washed in PBS twice ($240 \times g$ for 10 min), and resuspended in RPMI 1640 medium containing 4.5 g/L glucose supplemented with 2 mM L-glutamine, penicillin/streptomycin (50 IU/mL and 50 μg/mL, respectively) and 10% (v/v) fetal bovine serum (FBS).

2.3. Cell culture and incubation

The human hepatocellular carcinoma (HepG2) cells from the American Type Culture Collection (ATCC) were subcultured in a 75 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, penicillin/streptomycin (50 IU/mL and 50 μg/mL, respectively) and 10% (v/v) FBS. HepG2 cells and PBMC were maintained at 37 °C in a 5% CO$_2$/air incubator (Thermo Electron Co.) and verified in an inverted microscope (Nikon Eclipse Ti, Japan). To analyze the cytotoxicity effects of AuNPs, HepG2 cells and PBMC were incubated with AuNps-citrate and AuNps-PAMAM. Control experiments containing only PAMAM in the culture medium have also been performed. Cytotoxicity was investigated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. The genotoxicity was measured by the alkaline comet assay. Viability of the cells exposed to AuNps was also determined by the trypan blue exclusion assay, immediately before all the assays (Freshney, 2000). In a viable cell, trypan blue dye (Sigma–Aldrich, USA) is not absorbed. The number of viable cells was always >90% for each cell suspension in both control and treated groups before the assays.

2.4. Cytotoxicity assay

Cytotoxicity was also determinated using MTT assay (Mosmann, 1983), a method for determining cell viability by measuring the mitochondrial dehydrogenase action. This enzyme reduces MTT to water-insoluble blue formazan crystals. Cells were counted and plated (1×10^5 cells/well) in 96-well culture plates and allowed to adhere (HepG2) or stabilization (PBMC) at 37 °C in a 5% CO$_2$ atmosphere for 24 h. The freshly prepared AuNps-PAMAM and AuNps-citrate were dispersed in cell culture medium, diluted at concentrations from 0.01 to 50.0 μM and were added to each culture well. Doxorubicin (DXR) was used as the positive control and analyzed at the concentration of 0.3 μM. DXR is an antitumor agent that acts by intercalating the DNA. It is rapidly taken up into the nucleus of cells, inhibiting DNA synthesis, binding with high affinity to DNA by classical intercalation between base pairs, promoting single strand breaks in DNA and inhibiting DNA topoisomerase II (Cutts et al., 2005). A negative control containing only cells in culture medium was also evaluated. After 24 h of treatment, the plates were incubated with 10 μL of MTT (Sigma–Aldrich, USA) solution (0.5 mg/mL) for 4 h at 37 °C in a 5% CO$_2$ atmosphere. The plate was centrifuged at 1500 rpm for 10 min. The medium was removed and replaced by 100 μL of dimethyl sulfoxide (DMSO), followed by mixing to dissolve the formazan crystals. Absorbance was measured at 570 nm on a microenzyme-linked immunosorbent assay (ELISA) reader (Spectramax, Molecular Devices®) and the reduction of cell viability was expressed as the percentage compared with the negative control group designated as 100%. A control experiment carried out using only PAMAM in the culture medium did not induced cytotoxicity (data not shown).

2.5. DNA damage assay

Nanoparticle-induced DNA damage was performed by the comet assay (also referred to as the single-cell gel electrophoresis – SCGE analysis) under alkaline conditions (Singh et al., 1988). The negative control was exposed without AuNps under the same conditions. HepG2 cells and PBMC were cultured in 12-well culture plates as described above, and then pretreated for 3 h with 1.0 and 50.0 μM of AuNps-citrate and AuNps-PAMAM. Microscope slides were prepared in duplicate and coated with 1% normal melting point agarose (NMA). 60 μL of each cell suspension with 300 μL of low melting point agarose 1% (LMPA) were placed on these microscope slides containing NMA, deposited over the agarose layer. Coverslips were placed on the gels, which were left to set on ice. After gently removing the coverslips, the slides were immediately submersed in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Tritron X-100, pH 10) for 12 h in the dark. DNA was then allowed to unwind for 20 min in alkaline electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH > 13). Electrophoresis was performed under 25 V and 300 mA for 20 min. Subsequently, the slides were placed in a cold neutralizing buffer (400 mM Tris buffer, pH 7.5) for 15 min, dried in 100% methanol for 5 min, and stained with 50 μL of 20 μg/mL ethidium bromide in the dark. At least 50 comets per slide were analyzed under a fluorescence microscope (Nikon Eclipse E200, Japan) equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm, connected to a digital camera (Nikon DS Qi1, Japan). The classical visual analysis scoring of the comet assay was analyzed by a single analyst, in order to minimize scoring images variation. Data were based on 150 cells for each test or control that were visually scored as belonging to one of five classes, according to tail size and intensity. Classes 0, 1, 2, 3, or 4 were given, with 0 = no detectable damage and 4 = maximum damage. The damage index was obtained by the formula, damage index = $(0 \times n0) + (1 \times n1) + (2 \times n2) + (3 \times n3) + (4 \times n4)$. The variables n0–n4 represent the number of nucleoids with 0–4 damage level, and each experiment was performed in triplicate.

2.6. Cellular uptake

The AuNps cellular uptake was investigated using flow cytometry (Suzuki et al., 2007). In 6-weel cell culture plates, 1×10^6 cells/well were seeded and cultured for 24 h for attach (HepG2) or stabilization (PBMC), treated with 1.0 and 50.0 μM AuNps-PAMAM and AuNps-citrate concentrations at 37 °C in a 5% CO$_2$ atmosphere for 24 h. Cells were then harvested, washed and resuspended in PBS. The uptake of AuNps was analyzed by flow cytometer (FACSCalibur, BD BioSciences, San Jose, USA).

2.7. ROS intracellular generation

Intracellular generation of ROS was determined using oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Sigma–Aldrich, USA) as previously described by Sohaebuddin et al. (2010). A DCFH-DA assay was performed for untreated cells (negative control) and compared to HepG2 cells and PBMC treated with AuNps-citrate and AuNps-PAMAM, both at 1.0 and 50.0 μM concentrations. A positive control with hydrogen peroxide was included. After 24 h of exposure to AuNps, the cells were incubated in the presence of 10 μM of DCFH-DA for 30 min at 37 °C. Nonfluorescent DCFH-DA is rapidly oxidized to highly fluorescent 2′,7′-dichlorodihydrofluorescein (DCF) by ROS. Fluorescence from oxidized DCF was determined by FACSCalibur® flow cytometer equipped with a 488 nm laser. Data were taken from 10,000 cells per sample.

2.8. Statistical analysis

All experiments were carried out in triplicate, and the results were expressed as mean ± standard deviation of three independent experiments. Data were evaluated by one-way analysis of variance (ANOVA) followed by post hoc Tukey's Multiple Comparison Test, using Graph Pad Prism program software version 5. The results were considered statistically significant when $p < 0.05$.

3. Results

The typical TEM images and size distribution of the nanoparticles are shown in Fig. 1(a) for AuNps-PAMAM and (b) AuNps-citrate. The average diameter of AuNps-PAMAM and AuNps-citrate were
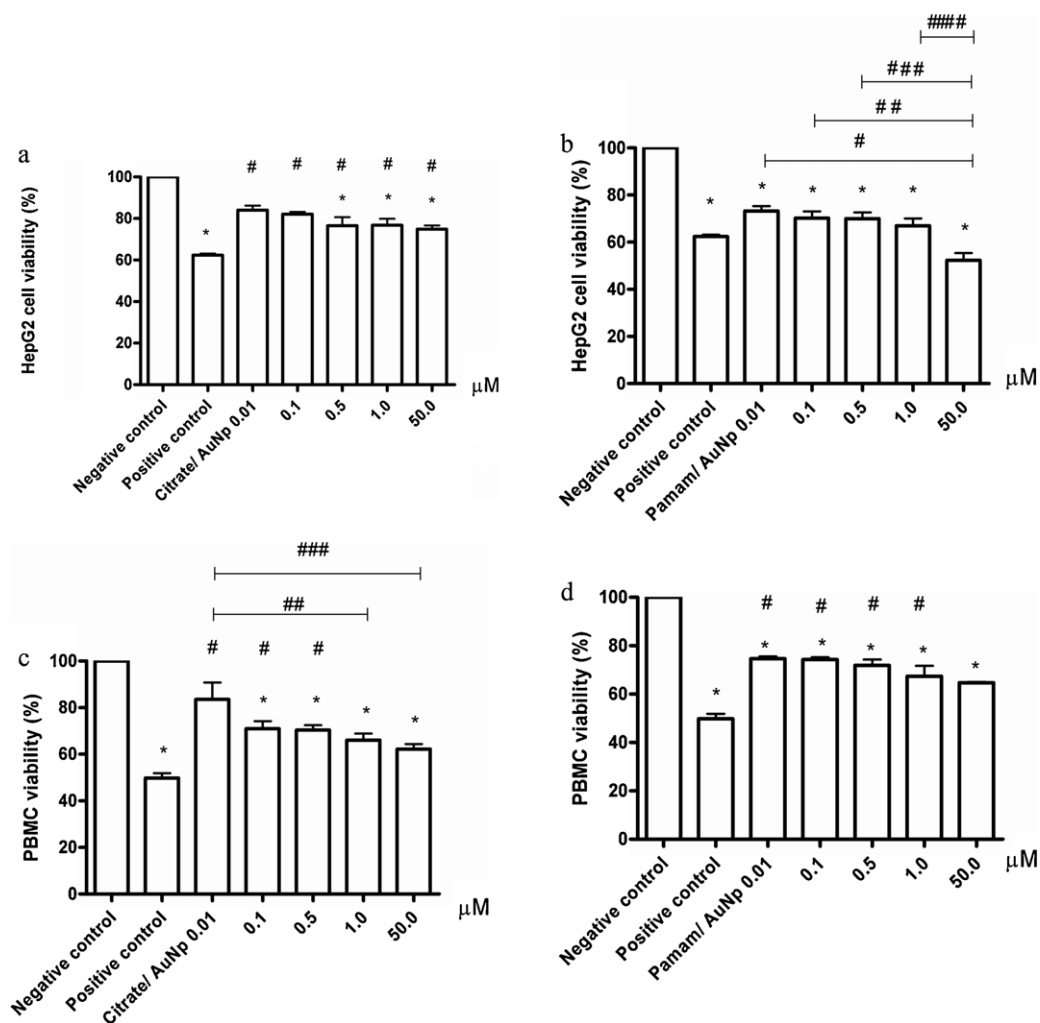
Fig. 2. (a) Percentage of living HepG2 cells after 24 h-incubation with AuNps-Citrate. Data are reported as means ± standard deviation (SD). *Statistically different from the negative control group (ANOVA, Tukey's Multiple Comparison Test, $p < 0.05$). #Statistically different from the positive control group (ANOVA, Tukey's Multiple Comparison Test, $p < 0.05$). (b) Percentage of living HepG2 cells after 24 h-incubation with AuNps-PAMAM. Data are reported as means ± standard deviation (SD). *Statistically different from the negative control group (ANOVA, Tukey's Multiple Comparison Test, $p < 0.05$). #,##,### and ####Statistically different between PAMAM/AuNp 0.01, 0.1, 0.5, 1.0 μM group, respectively, vs. 50.0 μM group (ANOVA, Tukey's Multiple Comparison Test, $p < 0.05$). (c) Percentage of living PBMC after 24 h-incubation with AuNps-citrate. Data are reported as means ± standard deviation (SD). *Statistically different from the negative control group (ANOVA, Tukey's Multiple Comparison Test, $p < 0.05$). #Statistically different from the positive control group (ANOVA, Tukey's Multiple Comparison Test, $p < 0.05$). ##Statistically different between Citrate/AuNp 0.01 μM group vs. 1.0 μM group (ANOVA, Tukey's Multiple Comparison Test, $p < 0.05$). ###Statistically different between Citrate/AuNp 0.01 μM group vs. 50.0 μM group (ANOVA, Tukey's Multiple Comparison Test, $p < 0.05$). (d) Percentage of living PBMC after 24 h-incubation with AuNps-PAMAM. Data are reported as means ± standard deviation (SD). *Statistically different from the negative control group (ANOVA, Tukey's Multiple Comparison Test, $p < 0.05$). #Statistically different from the positive control group (ANOVA, Tukey's Multiple Comparison Test, $p < 0.05$).

estimated using dynamic light scattering (DLS) analysis. Zeta potential and hydrodynamic diameter were measured before and after AuNps dilution into cell culture medium supplemented with serum (10% FBS) (Table 1).

After incubation of HepG2 cells and PBMC with AuNps-citrate and AuNps-PAMAM at concentrations from 0.01 to 50.0 μM for 24 h, cell viability was determined by MTT assay. As shown in Fig. 2, the viability of HepG2 cells (Fig. 2(a), AuNps-citrate and Fig. 2(b), AuNps-PAMAM) and PBMC (Fig. 2(c), AuNps-citrate and Fig. 2(d), AuNps-PAMAM) decreased significantly when compared to negative control ($p < 0.05$), except at 0.01 μM for AuNps-citrate to both cells. At the highest concentration (50.0 μM), we observed a substantial viability reduction in HepG2 cells and PBMC, both with respect to the negative control.

To investigate the DNA damage caused by both types of AuNps, the comet assay was performed upon incubation of the cells with 1.0 and 50.0 μM of citrate- and PAMAM-capped Nps. Tables 2 and 3 depict the extensive damage to DNA after treatment of HepG2 and

PBMC cells, respectively, with both AuNps. The damage index for AuNps-citrate at 50.0 μM and AuNps-PAMAM at 1.0 and 50.0 μM in HepG2 cells were statistically significant ($p < 0.05$), whereas AuNps-citrate at 1.0 μM did not show a significant effect ($p > 0.05$). Furthermore, the damage index for AuNps-citrate and AuNps-PAMAM at 1.0 μM did not show a significant effect ($p > 0.05$) for PBMC. At a concentration of 50.0 μM, the AuNps-PAMAM induced a significant toxic effect ($p < 0.05$) on PBMC cells, compared to the negative control.

ROS and reactive nitrogen species (RNS) are generated during the inflammatory response, especially by phagocytes, and they may contribute to the pathology of many inflammatory conditions (Paino et al., 2011). Furthermore, they represent a disturbance in the balance between pro-oxidant/antioxidant reactions. AuNps cellular uptake were acquired by flow cytometry and appear in Table 4 as a function of side scatter (SSC), representing the cell granularity, and forward scatter (FSC), representing the cell size. A significant increase in the SSC values was observed for HepG2 cells only for

Table 2
DNA damage index obtained by comet visual score in HepG2 cells pretreated with Citrate coated AuNps or PAMAM coated AuNps and the negative control.

Treatments (μM)	Comet	Classes					Damage index ± SD	Frequency (%)
	0	1	2	3	4			
Negative control	96	30	12	9	3		31 ± 11.35	36.0
AuNps-citrate (1)	66	49	19	3	0		32 ± 3.60	47.3
AuNps-citrate (50)	50	57	30	13	0		52 ± 6.24[*],[#]	66.6
AuNp-PAMAM (1)	55	33	35	26	1		61.6 ± 5.03[*]	63.3
AuNp-PAMAM (50)	50	34	31	30	5		68.6 ± 10.9[*]	66.6

Data are reported as means ± standard deviation (SD) of triplicate experiments. One hundred fifty nucleoids were analyzed per treatment in HepG2 cells.
[*] Statistically different from the negative control without AuNps (ANOVA, Tukey's Multiple Comparison Test, $p < 0.05$).
[#] Statistically different between Citrate/AuNp 1.0 μM group vs. Citrate/AuNp 50.0 μM group (ANOVA, Tukey's Multiple Comparison Test, $p < 0.05$).

Table 3
DNA damage index obtained by comet visual score in PBMC pretreated with Citrate coated AuNps or PAMAM coated AuNps and the negative control.

Treatments (μM)	Comet	Classes					Damage index ± SD	Frequency (%)
	0	1	2	3	4			
Negative control	94	35	17	4	0		25.3 ± 3.5	37.3
AuNps-citrate (1)	63	45	23	27	2		50.0 ± 7.0	64.6
AuNps-citrate (50)	60	54	19	12	5		49.3 ± 27.5	60.0
AuNp-PAMAM (1)	60	33	24	4	0		51.3 ± 14.8	40.6
AuNp-PAMAM (50)	45	25	14	21	6		52.0 ± 13.1[*]	44.0

Data are reported as means ± standard deviation (SD) of triplicate experiments. One hundred fifty nucleoids were analyzed per treatment in PBMC.
[*] Statistically different from the negative control without AuNps (ANOVA, Tukey's Multiple Comparison Test $p < 0.05$).

Table 4
HepG2 and PBMC cellular uptake by AuNps-Citrate and AuNps-PAMAM after 24 h exposure using flow cytometry.

Treatments (μM)	HepG2 cells		PBMC	
	%FSC	%SSC	%FSC	%SSC
Negative control	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
AuNps-citrate (1)	99.2 ± 0.5	109.0 ± 3.3	102.1 ± 1.1	104.6 ± 3.0
AuNps-citrate (50)	100.7 ± 1.8	105.1 ± 4.1	98.45 ± 0.8	115.7 ± 3.9[*]
AuNp-PAMAM (1)	91.3 ± 2.0	96.8 ± 3.0	98.34 ± 0.5	128.4 ± 4.1[*]
AuNp-PAMAM (50)	85.28 ± 7.8	126.0 ± 0.3[*],[#]	98.46 ± 1.8	135.3 ± 2.1[*]

Data are reported as means ± standard deviation (SD) of triplicate experiments.
[*] Statistically different from the negative control without AuNps (ANOVA, Tukey's Multiple Comparison Test, $p < 0.05$).
[#] Statistically different between PAMAM/AuNp 1.0 μM group vs. PAMAM/AuNp 50.0 μM group (ANOVA, Tukey's Multiple Comparison Test, $p < 0.05$).

AuNps-PAMAM treated cells, at a concentration of 50.0 μM. On the other hand, PBMC incubated with citrate- and PAMAM-covered AuNps exhibited an increase ($p < 0.05$) in the SSC values for both concentrations investigated from the negative control, except at 1.0 μM AuNps-citrate. A statistically significant ($p < 0.05$) measurement of intracellular ROS was observed for both HepG2 and PBMC upon treatment with AuNps, as shown in Fig. 3a and b, respectively.

4. Discussion

Data from zeta potential analysis, as depicted in Table 1, suggests that cell culture media containing 10% FBS serum influences the nanoparticles stability. Since the medium contains a variety of salts, amino acids and vitamins, its high ionic strength decrease the electrostatic repulsive forces among the nanoparticles, inducing aggregation (Fatisson, 2012). On the other hand, proteins from
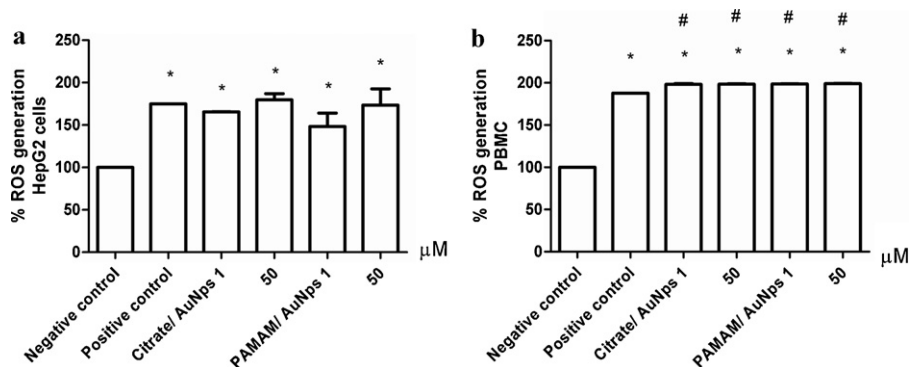

Fig. 3. (a) Effect of AuNp-citrate and AuNp-PAMAM on the generation of ROS in HepG2 cells. The %ROS generation of negative control was considered as 100%. Data are reported as means ± standard deviation (SD). *Statistically different from the negative control group (ANOVA, Tukey's Multiple Comparison Test, $p < 0.05$). (b) Effect of AuNp-citrate and AuNp-PAMAM on the generation of ROS in PBMC. The %ROS generation of negative control was considered as 100%. Data are reported as means ± standard deviation (SD). *Statistically different from the negative control group (ANOVA, Tukey's Multiple Comparison Test, $p < 0.05$). #Statistically different from the positive control group (ANOVA, Tukey's Multiple Comparison Test, $p < 0.05$).

serum in the medium can adsorb on the nanoparticles creating a protein "corona", resulting in the stabilization of the colloidal suspensions and preventing aggregation upon modifying their zeta potential (Fatisson, 2012; Chithrani et al., 2006), as observed here via DLS or hydrodynamic diameter (Table 1). The interaction between the cells and the nanoparticles could be mediated by nonspecific adsorption of serum proteins onto the gold surface, as proposed by Chithrani et al. (2006). In our case, the AuNp uptake mechanism may occur via receptor-mediated endocytic pathways (clathrin mediated), in agreement to what has been reported by Nativo et al. (2008).

Data from literature regarding the cytotoxicity and genotoxicity of citrate or PAMAM-coated AuNps are somehow conflicting (Patra et al., 2007; Pan et al., 2009). The controversy comes from the variability of parameters, including cell lines used in toxicity assays, concentrations, surface charge and coatings. For example, Connor et al. (2005) demonstrated non-toxic effects of Au nanospheres (diameter from 4 to 18 nm, covered with citrate, cysteine, glucose, biotin, and cetyltrimethylammonium bromide) on human leukemia cell line (K562) cells. On the other hand, Patra et al. (2007) reported that 13 nm citrate-coated AuNps exhibited toxicity against a human carcinoma lung cell line, at equal concentrations from 20 nM to 120 nM. As revealed here via cytotoxicity assays, both PAMAM-coated and citrate-coated AuNps induced cytotoxicity in HepG2 cells or PBMC. A decrease in cell viability upon incubation with AuNps-citrate and AuNps-PAMAM for both HepG2 and PBMC has been observed using the MTT assay. A change in morphology of HepG2 cells upon AuNps treatment also indicated the toxicity effects (data not shown).

The genotoxicity assays employed here, as shown in Table 2 (HepG2 cells) and Table 3 (PBMC), can be related to the nanometric dimensions of AuNps, which may undergo cell uptake (Lewinski et al., 2008). It was evidenced that AuNps-PAMAM and AuNps-citrate induced DNA damage, as an indicative of genotoxicity. This effect is related to the cellular toxicity of gold nanoparticles, which in our case is also related to the small size of the particles that easily undergo cell uptake through diffusion, in agreement with Pernodet et al. (2006). Li et al. (2008) demonstrated that serum coated 20 nm AuNps were also able to induce genotoxicity in the form of single-strand lesions in DNA in human lung fibroblasts. Our analyses provided convincing evidence of the toxic effect of AuNps, indicating that surface charge or size may be a major determinant of how AuNps impact cellular processes. Furthermore, the DNA damage index for AuNps-PAMAM and AuNps-Citrate was statistically significant analyzed for HepG2 cells, except at $1.0\,\mu M$ AuNps-Citrate. The genotoxicity for PBMC was statistically significant only upon incubation with AuNps-PAMAM at $50.0\,\mu M$. The tendency of the AuNps to accumulate in the cells nuclei was associated with their small size, which allows the nanoparticles to freely diffuse through pore complex (Zhao and Nalwa, 2007). Since the comet assay evaluates the reversible DNA damage, our genotoxicity results also suggest that PBMC, a primary cell culture, were less sensitive to DNA damage to a certain extent the nanoparticles than HepG2 cancer cells. The purpose was to analyze the repair system in comet assay evidencing the DNA repair.

The use of SSC parameter obtained via flow cytometry has been proposed as an efficient way to investigate cell uptake (Suzuki et al., 2007). In our analyses, the uptake of both types of AuNps was monitored by SSC (Table 4), revealing that for HepG2 cells, the relative SSC values were significantly increased ($p < 0.05$) only for cells incubated with AuNps-PAMAM at $50.0\,\mu M$. In contrast, the PBMC exhibited an increase in the SSC values for cells incubated with both types of nanoparticles at $50.0\,\mu M$. Furthermore, a significantly increase in SSC was also observed for PBMC upon incubation with AuNps-PAMAM at the lower concentration investigated ($1.0\,\mu M$).

The generation of ROS represents an important mechanism in the toxicity induced by AuNps, which may disturb the equilibrium between antioxidant and oxidant intracellular processes. The ROS generation was evidenced here for HepG2 cells and PBMC incubated with both types of nanoparticles for 24 h, as shown in Fig. 3a and b, respectively. For both PBMC and HepG2 cells, a significant increase in the ROS generation was observed upon incubation with citrate and PAMAM-covered AuNps (Fig. 3a and b). The cellular oxidative stress increased in both cell lines may be directly correlated with AuNps exposure, homologous to an increase in cytotoxicity.

5. Conclusion

Taken together, our findings suggest that the exposure of HepG2 cells and PBMC to AuNps-PAMAM and AuNps-citrate might lead to the disturbance of cells with cytotoxic effects and DNA damage. The correlation between the toxic effects of Au nanoparticles with their physico-chemical and surface properties may be an important step forward to the application of these nanomaterials in cancer treatment. Our results from the comet assay, for example, revealed that the immune system cells (PBMC) were less sensitive to DNA damage than cancer HepG2 cells, upon exposure to AuNps. The latter is an indicative that nanoparticulate systems may be applied in cancer therapy with reduced side effects in the future studies.

Conflict of interest

The authors declare that there is no conflict of interest regarding the work reported in this paper.

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