

OPEN ACCESS

Iron oxide nanoparticles show no toxicity in the comet assay in lymphocytes: A promising vehicle as a nitric oxide releasing nanocarrier in biomedical applications

To cite this article: R de Lima *et al* 2013 *J. Phys.: Conf. Ser.* **429** 012021

View the [article online](#) for updates and enhancements.

You may also like

- [Surface modification of magnetic nanoparticles in biomedicine](#)
Xin Chu, , Jing Yu et al.
- [Nitric oxide releasing iron oxide magnetic nanoparticles for biomedical applications: cell viability, apoptosis and cell death evaluations](#)
R de Lima, J L de Oliveira, A Ludescher et al.
- [Evidence of iron \(III\) reduction in -Fe₂O₃ nanoparticles due to meso-2,3-dimercaptosuccinic acid functionalization](#)
Eloiza S Nunes, Emilia C D Lima, Maria A G Soler et al.



ECS
The
Electrochemical
Society
Advancing solid state &
electrochemical science & technology

DISCOVER
how sustainability
intersects with
electrochemistry & solid
state science research

Iron oxide nanoparticles show no toxicity in the comet assay in lymphocytes: A promising vehicle as a nitric oxide releasing nanocarrier in biomedical applications

R de Lima¹, J L Oliveira¹, P S K Murakami², M A M Molina³, R Itri³, P Haddad²
and A B Seabra^{2*}

¹Departamento de Biotecnologia, Universidade de Sorocaba, Rodovia Raposo Tavares Km 92, CEP 18023-000, Sorocaba, SP, Brazil

²Departamento de Ciências Exatas e da Terra, Universidade Federal de São Paulo, Rua São Nicolau 210, CEP 09913-030, Diadema, SP, Brazil

³Instituto de Física, Universidade de São Paulo, CP 66318, CEP 05314-970, SP, Brazil

*E-mail: amedeia.seabra@unifesp.br

Abstract. This work reports the synthesis and toxicological evaluation of surface modified magnetic iron oxide nanoparticles as vehicles to carry and deliver nitric oxide (NO). The surface of the magnetic nanoparticles (MNPs) was coated with two thiol-containing hydrophilic ligands: mercaptosuccinic acid (MSA) or dimercaptosuccinic acid (DMSA), leading to thiolated MNPs. Free thiols groups on the surface of MSA- or DMSA-MNPs were nitrosated leading to NO-releasing MNPs. The genotoxicity of thiolated-coated MNPs was evaluated towards human lymphocyte cells by the comet assay. No genotoxicity was observed due to exposure of human lymphocytes to MSA- or DMSA-MNPs, indicating that these nanovectors can be used as inert vehicles in drug delivery, in biomedical applications. On the other hand, NO-releasing MNPs showed genotoxicity and apoptotic activities towards human lymphocyte cell cultures. These results indicate that NO-releasing MNPs may result in important biomedical applications, such as the treatment of tumors, in which MNPs can be guided to the target site through the application of an external magnetic field, and release NO directly to the desired site of action.

1. Introduction

Nitric oxide (NO) is a key molecule involved in several important physiological processes, such as the dilation of blood vessels, inhibition of platelet adhesion and aggregation, cell communication, wound healing, immune responses, apoptosis and anti-cancer activities, among others [1]. NO is a gaseous free-radical and one of the smallest endogenous molecules with the ability to function as a chemical messenger, particularly in cells of the vascular endothelium and immune system. Medical and scientific interest in NO has grown exponentially since 1992, when it was nominated “Molecule of the Year” [2]. Its documented physiological impacts are ever expanding, and NO is now known as one of the most important mediators of intra- and extracellular processes and is one of the major targets of the pharmaceutical industry [3]. Administration of high doses of NO (micro and milli concentrations) are known to play a key role in the immunological system and thus to initiate tumor regression [4].

Although NO is used in many biomedical applications, its utility is limited by its short half-life and instability during storage. As a free radical, in the biological medium, NO can readily react with biomolecules, such as hemoglobin, leading to its inactivation. Therefore, there is great interest in the development of NO-releasing drugs and matrices which are capable of stabilizing and releasing NO locally, directly into different tissues and organs [5]. Among the nanostructured materials aimed at drug delivery, magnetic nanoparticles (MNPs), in particular iron oxide nanoparticles, such as magnetite (Fe_3O_4), are considered a promising material for biomedical applications [6]. Indeed, MNPs have been used for targeted drug delivery to specific cells, tissues or organs, for enhancing magnetic resonance imaging contrast and for hyperthermia treatments [7]. In drug delivery, iron oxide MNPs can be easily transported *in vivo* to the desired site of action by application of an external electrical-magnetic field. Magnetization disappears upon removal of the magnetic field, leading the iron oxide MNPs to remain at the target site for a certain period [8].

However, in order to propose the use of MNPs in biomedical applications, such as vehicles for drug delivery, it is mandatory to investigate the toxicological aspects of these nanomaterials. In this context, nanomaterial safety, in particular nanogenotoxicology, is an increasing issue in the field of nanotechnology [9,10]. Recent papers highlighted that many nanomaterials induce cytotoxicity and oxidative stress. Conflicting data have been reported [9,10]. Therefore, there is great interest in the characterization of possible toxic effects of nanomaterials, in particular MNPs, aimed for pharmacological applications. In this context, this work reports the synthesis and evaluation of genotoxicity of surface-modified MNPs, as vehicles to carry and deliver NO in biomedical applications.

2. Methods

2.1. Synthesis of magnetic nanoparticles (MNPs)

A co-precipitation method was used to synthesize MNPs [11]. Briefly, 4.0 mL of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution and 1.0 mL of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ solution (molar ratio 2:1) in 1.0 mol/L HCl were mixed and stirred, while a volume of 50 mL of NH_4OH (0.7 mol/L) was added as precipitator. The solution was centrifuged and the precipitate was decanted, followed by the addition of 6.0 mL of oleic acid. This mixture was stirred for 20 minutes. The solution was centrifuged several times and the new precipitate was washed several times with ethanol and acetone, leading to MNPs covered with oleic acid.

Oleic acid coated-MNPs (~10.0 mg) were suspended in 1.0 mL of toluene while mercaptosuccinic acid (MSA) or 2,3 dimercaptosuccinic acid (DMSA) (molar ratio Fe_3O_4 :MSA or DMSA = 1:40) was dissolved in dimethyl sulfoxide (DMSO) in a second tube. The solutions were mixed and vigorously stirred for 14 h producing a black powder that was isolated by centrifugation. This procedure led to ligand exchange and, hence, to the formation of water stable thiol-containing MNPs (SH-NPs).

2.2. Nitrosation of thiolated magnetic nanoparticles

The thiol groups (SH) on the surface of MSA- or DMSA-coated MNPs were nitrosated by sodium nitrite (NaNO_2) leading to the formation of S-nitroso-MNPs, which are spontaneous NO donors. In this step, filtered MSA- or DMSA-MNPs were suspended in deionised water. A volume of 200 μL of aqueous sodium nitrite (60 mmol/L) was added to the MNPs. After 15 min of incubation at room temperature, the nanoparticle suspension was filtered by centrifugal ultrafiltration by using a Microcon centrifugal filter device containing ultrafiltration membranes (MWCO 10-kDa molar mass cut-off filter, Millipore, Billerica, MA, USA) and washed with deionised water to remove excess unreacted nitrite.

2.3. Cell description

For the analyses involving lymphocytes, the cells were separated from whole blood using Ficoll-Paque™ PLUS medium (GE Healthcare, Little Chalfont, UK). The blood was provided by donors aged between 18 and 24 years (who freely signed terms of agreement forms) and the project was approved by the Ethics Committee of the University of Sorocaba (protocol #008/08). Blood samples were collected using disposable materials throughout the procedure. The lymphocytes were placed in Roswell Park Memorial Institute (RPMI) 1600 culture medium (Cultilab) containing 300 µg/mL of L-glutamine and 200 µg/mL of NaHCO₃, supplemented with 5% bovine fetal serum, 50 µg/mL of gentamicin sulfate (antibiotic) and 2 µg/mL of amphotericin B (antifungal). The culture was kept at 37°C, under a humidified atmosphere containing 5% CO₂.

2.4. Incubation of human lymphocytes with MNPs

Human lymphocytes were incubated with MSA- or DMSA-MNPs (thiol-containing MNPs, as control groups) and nitrosated-MSA (SNO-MSA) or nitrosated-DMSA (SNO-DMSA)- MNPs at the following nanoparticle concentrations: 0.01; 0.1 and 0.5 mg/mL, for 1 and 24 h. Negative and positive controls employed phosphate buffered saline (PBS) and H₂O₂ (200 µmol/L), respectively.

2.5. Comet assay

Each treatment group involved 10 µL of lymphocytes in 110 µL of low melting point agarose (0.6%) and the mixtures were placed onto microscope slides that had been pre-coated with normal melting point agarose (1.5%). Coverslips were positioned over the materials, and the slides were placed in a refrigerator for polymerization. After polymerization, the coverslips were removed, and the slides were treated for 90 minutes with an ice-cold (4°C) lysis solution (2.5 mol/L NaCl, 0.1 mol/L ethylenediaminetetraacetic acid (EDTA), 10 mmol/L tris(hydroxymethyl)aminomethane (Tris), 1% Triton X-100TM, pH 10). All groups were incubated in an electrophoresis buffer (0.3 mol/L NaOH, 1 mmol/L EDTA, pH 13) for 20 minutes, followed by electrophoresis for 20 minutes at 1.3 V/cm. After electrophoresis, the slides were covered with a neutralizing solution (0.4 mol/L Tris, pH 7.5) for 5 min, washed three times with distilled water, and allowed to rest overnight at room temperature. Prior to staining, the dry slides were left in a fixing solution (15% w/v trichloroacetic acid, 5% (w/v) zinc sulfate, and 5% glycerol) for 10 minutes, and were further washed three times with distilled water. After these procedures, the slides were allowed to rest at room temperature for 1.5 hours. They were then rehydrated with distilled water and stained for approximately 15 minutes with silver staining solution consisting of 34 mL of solution A (0.2% w/v ammonium nitrate, 0.2% w/v silver nitrate, 0.5% w/v tungstosilicic acid, 0.15% v/v formaldehyde, and 5% w/v sodium carbonate) and 66 mL of solution B (5% sodium carbonate), followed by a bath in distilled water and a bath in stop solution. Lastly, the slides were further washed with distilled water and allowed to dry at room temperature. Staining using silver is analogous to fluorescence, where the positive charge of the silver enables it to bind with DNA and DNA fragments, producing the characteristic color. Throughout the procedures involving cellular material, both natural light and light from fluorescent lamps were avoided to prevent possible influence on the results.

Analyses were performed using the Zeiss Axovert optical microscope and at least 100 cells were counted on each slide, with 3 slides for each test (around 300 cells). The experiment was performed in triplicate, giving a total of 900 cells analysed for each sample tested. The comet assay analyses were performed by assigning a score of 0 to 4, according to the quantity of DNA in the tail, and the length of the tail: Class 0 corresponded to intact cells, Class 1 corresponded to cells with minimal damage; Class 2 to average damage; Class 3 to severe damage; and Class 4 to cells with maximum damage [12]. For this visual method, the number of cells found for each score was multiplied by the value of the score and the values were summed at the end of the analysis of each slide. Since the score depended on the number of cells observed, an index of tail damage (TD) was created by dividing the score given to the slide by the number of cells analysed on the slide.

3. Results and Discussion

3.1. Synthesis of MSA- or DMSA-coated MNPs

Iron oxide MNPs comprised by Fe_3O_4 were coated with two thiol-containing hydrophilic ligands: MSA or DMSA (Figure 1).



Figure 1. Chemical representation of MSA (i) and DMSA (ii) used as coating for MNPs.

Figure 2 represents schematically the nanoparticle preparation. The presence of thiol-containing ligands (MSA or DMSA) on the nanoparticle surface is responsible to increase water-solubility of the nanoparticles, to provide thermal stabilization, and to avoid nanoparticle aggregation, in addition, it represents an important site for bioconjugation. Indeed, in this work, free thiols groups (SH) on the nanoparticle surfaces were used to load NO to the nanoparticle.

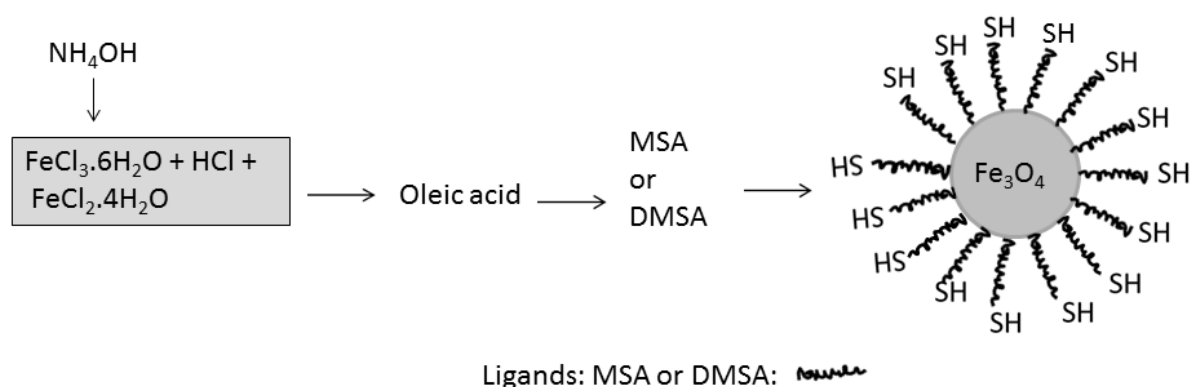


Figure 2. Schematic representation of the synthesis of MSA- or DMSA-coated MNPs, leading to the formation of thiol-containing nanoparticles.

3.2. Nitrosation of thiols groups on MSA- or DMSA-coated MNPs

Thiol groups on MSA- or DMSA-coated nanoparticles were nitrosated by the addition of sodium nitrite, leading to the formation of S-nitroso-MNPs (SNO-MNPs) (Figure 3). In acidified aqueous solution, nitrite (NO_2^-) is in equilibrium with nitrous acid (HNO_2), which is considered the nitrosating agent of -SH groups leading to the formation of -SNO [13]. SNO moieties act as spontaneous NO donors, due to homolytic S-N bond cleavage, with free NO release [13], as represented in Figure 3.

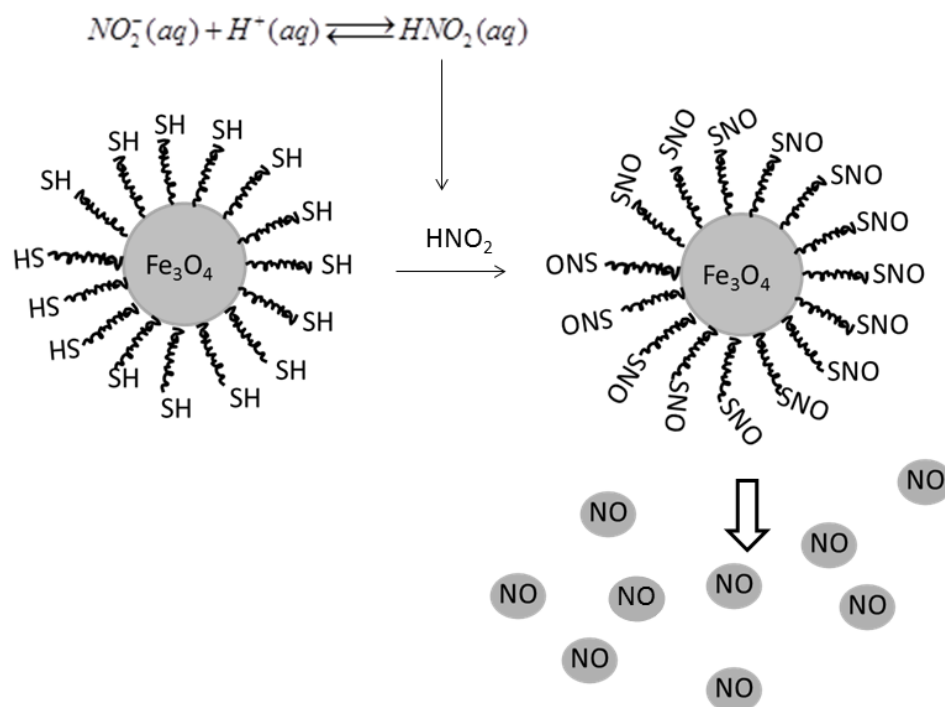


Figure 3. Schematic representation of nitrosation of thiols groups of MSA- or DMSA-MNPs upon incubation with nitrite, leading to the formation of SNO-MNPs, which are spontaneous NO donors.

3.3. Genotoxicity of MNPs (Comet Assay)

The genotoxicities of thiolated and nitrosated MNPs toward human lymphocytes were evaluated by comet assay that detects DNA single strand breaks, alkaline labile sites, and excision processes in individual cells. Figure 4 shows the DNA damage (as expressed in % tail DNA content) due to exposure of lymphocytes to MSA-MNPs (thiolated NPs) and SNO-MSA-MNPs (S-nitroso-MSA-MNPs) for 1 and 24 h, at different nanoparticle concentrations (0.5; 0.1 and 0.01 mg/mL), as indicated in the Figure. As can be observed, MSA-MNPs were found to be not genotoxic at all tested concentrations, after 1 and 24 h of exposure to human lymphocytes (Figure 4). The values of tail DNA were shown to be similar to the values obtained for the negative control, indicating the absence of genotoxicity of these MNPs.

However, NO-releasing MNPs (SNO-MSA-MNPs) were found to be genotoxic, under the same experimental conditions (Figure 4). The genotoxicity was higher after 24 h of cell exposition, in comparison to 1 h of exposure, indicating a time-dependence toxicity of NO-releasing MNPs. No concentration-dependence was observed for SNO-MSA-MNPs, since the values of DNA damage were similar for all tested MNPs concentrations.

Figure 5 shows the DNA damage due exposure of lymphocytes to DMSA-MNPs (thiolated NPs) and SNO-DMSA-MNPs (S-nitroso-DMSA-MNPs) for 1 and 24 h, at different nanoparticle concentrations, as indicated in the Figure. MSA-MNPs exhibited low genotoxicity. Although, the intensity of DNA damage was found to be discrete, a slight toxicity was observed for DMSA-MNPs at all concentrations studied, after 1 and 24 h of exposition. By comparing the genotoxicity of MSA-MNPs (Figure 4) with DMSA-MNPs (Figure 5) it can be noted that MSA-coated MNPs are less genotoxic in comparison with DMSA-MNPs, although both nanoparticles can be considered not toxic to cells.

On the other hand, the genotoxicity of SNO-DMSA-MNPs was higher related to DMSA-NPs. As expected, the presence of NO on NP surface led to toxic effects. Moreover, the DNA damage depended on the time of exposure, since 24 h of cell incubation with SNO-DMSA-MNPs resulted in a

more intensive genotoxicity, compared with exposure of only 1 h. SNO-DMSA-MNPs at different concentrations caused similar DNA damage (Figure 5).

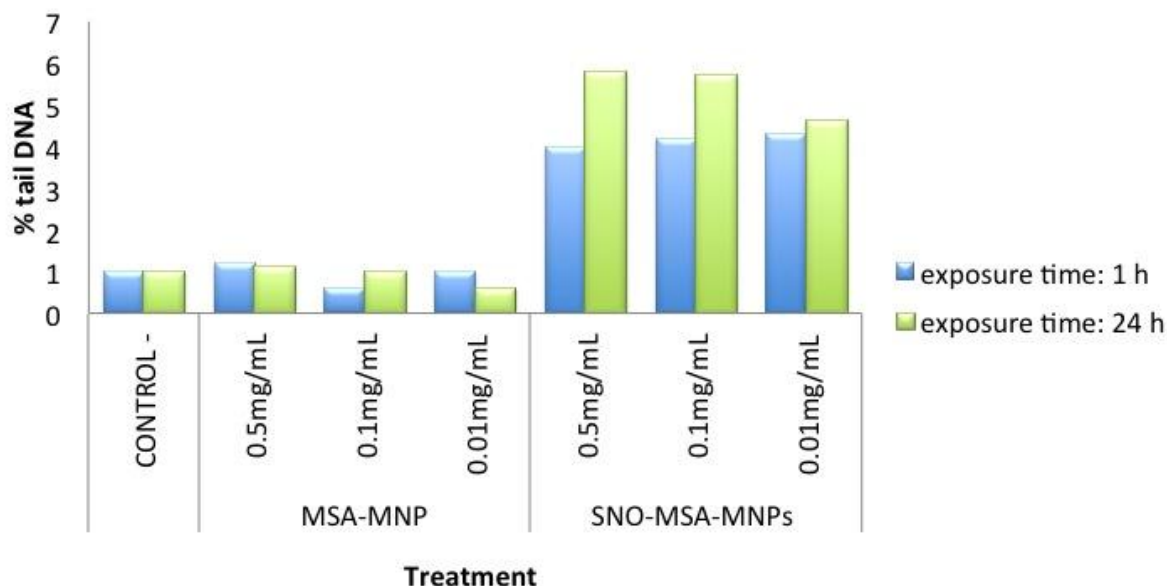


Figure 4. Results of DNA tail damage obtained for human lymphocyte cells after treatments with: MSA-MNP (MSA-coated magnetic nanoparticles) and SNO-MSA-MNP (S-nitrosated MSA-coated magnetic nanoparticles) at concentrations 0.5, 0.1 and 0.01 mg/mL. Exposure time: 1 and 24 h, as indicated in the Figure.

Uncoated iron oxide MNPs were found to have toxic effects towards cells [14]. However, MNPs toxicity can be greatly reduced by coating their surface [14]. In this context, this report shows that MSA- and DMSA-coated iron oxide MNPs are not genotoxic towards human lymphocytes. This result can be understood by assuming that both MSA and DMSA (the later to a lesser extend) are responsible to create a barrier for direct contact between the MNPs and the exposed cell, inhibiting possible toxic effects. Therefore, MSA- or DMSA-coated MNPs offer a high potential in biomedical applications, in particular, in drug delivery.

Both SNO-MSA-MNPs and SNO-DMSA-MNPs exhibited genotoxic effects on lymphocytes, under the experimental conditions employed in this study. Indeed, NO can both promote and inhibit cell damage, apoptosis, acting as anti- or pro- oxidant agents [1,4]. The biological roles of NO are greatly influenced by its source of production, concentration and flux. In fact, large concentrations of NO (i.e., micromolar) produce reactive nitrogen species, which along with reactive oxygen species result in oxidative and nitrosative stress, leading to DNA base deamination, nitrosylation/nitrosation of enzymes, impaired cellular function, enhanced inflammatory reactions, inhibited mitochondrial respiration and cell apoptosis [1,4]. On the other hand, lower NO concentrations (i.e., nano to picomolar) present anti-apoptotic effects, promote angiogenesis, wound healing and inhibition of platelet adhesion [1,4]. The concentrations used in this present work (0.01 to 0.5 mg of SNO-MNPs/mL) were high enough to promote genotoxic effects towards cells.

Based on the results reported here, NO-releasing MNPs might find important application against tumor cell lines. Indeed, in cancer science, NO therapies have been tested against numerous healthy and tumor cell lines, revealing that susceptibility depends on concentration, cell type, and the oxygen environment surrounding the site of NO delivery [4]. Due to the ability to have genotoxic effects against cells, SNO-MNPs can be used to carry and to delivery NO directly to the target site, since the MNPs can be guided to tumors cells, upon application of an external magnetic field. These nanoparticles would be able to release NO direct to cancer cells, where NO has its toxic effects. At the

present moment, toxicological evaluations of NO-releasing MNPs towards tumors cell lines are under progress.

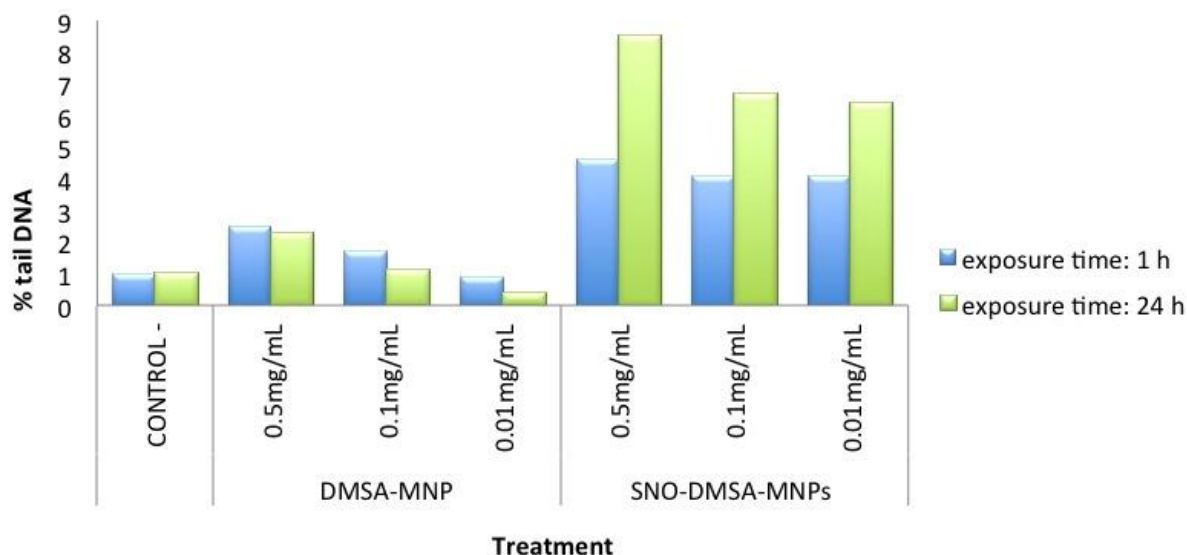


Figure 5. Results of DNA tail damage obtained for human lymphocyte cells after treatments with: DMSA-MNP (DMSA-coated magnetic nanoparticles) and SNO-DMSA-MNP (S-nitrosated DMSA-coated magnetic nanopartilces) at concentrations 0.5, 0.1 and 0.01 mg/mL. Exposure time: 1 and 24 h, as indicated in the Figure.

4. Conclusions

This work describes the genotoxicity of thiolated and nitrosated iron oxide MNPs. Thiol-coated MNPs showed no genotoxicity towards human lymphocyte cells, while nitroso-MNPs were found to lead to toxic effects on the cells, under the same experimental conditions. These results indicate the promising use of MSA or DMSA for coating MNPs aimed for biomedical applications, such as drug delivery. Moreover, due to the genotoxicity of SNO-MNPs, these nanoparticles might find important applications in cancer treatment, since these nanomaterials could be employed to deliver high amounts of NO directly to the target site of application, where NO can have its cytotoxic effects.

5. Acknowledgements

FAPESP, CNPq, CAPES, INOMAT (MCT/CNPq) and Brazilian Network in Nanotoxicology (MCT/CNPq). The authors would like to thanks Prof. Mauricio S. Baptista (IQ-USP) for the use of laboratory facilities and Prof. Carol H.Collins for revising the manuscript.

6. References

- [1] Carpenter A W and Schoenfish M H 2012 *Chem. Soc. Rev.* **41** 3742
- [2] Cullota E and Koshland Jr. 1992 *Science* **258** 1861
- [3] Serafim R A M, Primi M C, Trossini G H G and Ferreira E I 2012 *Curr. Med. Chem.* **19** 386
- [4] Choudhari S K, Sridharan G, Gadail A and Poornima V 2012 *Oral Oncol.* **48** 475
- [5] Seabra A B and Duran N 2010 *J. Mat. Chem.* **20** 1624
- [6] Majewski P and Thierry B 2007 *Crit. Rev. Solid State* **32** 203

- [7] Mahmoudi M, Sant S, Wang B, Laurent S, Sen T, Lord O T, Walter M J, Dasgupta R, Walker D and Clark S M 2011 *Adv. Drug Delivery Rev.* **63** 24
- [8] Klostergaard J and Seeney CE 2012 *Nanomed. Nanotechnol. Biol. Med.* **8** S37
- [9] Li Y-F and Chen C 2011 *Small* **7** 2965
- [10] Mahmoudi M, Hofmann H, Rothen-Rutishauser R and Petri-Fink A 2012 *Chem. Rev.* **112** 2323
- [11] Cha Y J, Kim M J, Choa Y H, Kim J, Nam B, Lee J, Kim D H and Kim K 2010 *IEEE Trans. Magn.* **46** 443
- [12] Lima R, Feitosa L, Maruyama C R, Barga M A, Yamawaki P C, Vieira I J, Teixeira E M, Corrêa A C, Mattoso L H C and Fraceto L F 2012 *Int. J. Nanomed.* **7** 3555
- [13] Seabra A B, Martins D M, da Silva R, Simões M M S G, Brocchi M and de Oliveira MG 2010 *Artif. Organs* **34** E204
- [14] Auffan M, Decome L, Rose J, Orsiere T, De Meo M, Briois V, Chaneac C, Olivi L, Le Franc J L B, Botta A, Wiesner M and Bottero J Y 2006 *Environ. Sci. Technol.* **40** 4367