Nitric oxide releasing iron oxide magnetic nanoparticles for biomedical applications: cell viability, apoptosis and cell death evaluations

R de Lima¹, JL de Oliveira¹, A Ludescher², MM Molina³, R Itri³, AB Seabra² and PS Haddad²*

¹ Departamento de Biotecnologia, Universidade de Sorocaba, CEP 18023-000, Sorocaba, S.P., Brazil
² Departamento de Ciências Exatas e da Terra, Universidade Federal de São Paulo, Diadema, SP, Brazil
³ Instituto de Física, Universidade de São Paulo, CEP 05314-970, São Paulo, SP, Brazil

*E-mail: haddadps@gmail.com

Abstract. Nitric oxide (NO) is involved in several physiological and pathophysiological processes, such as control of vascular tone and immune responses against microbes. Thus, there is great interest in the development of NO-releasing materials to carry and deliver NO for biomedical applications. Magnetic iron oxide nanoparticles have been used in important pharmacological applications, including drug-delivery. In this work, magnetic iron oxide nanoparticles were coated with thiol-containing hydrophilic ligands: mercaptosuccinic acid (MSA) and dimercaptosuccinic acid (DMSA). Free thiol groups on the surface of MSA- or DMSA- coated nanoparticles were nitrosated, leading to the formation of NO-releasing iron oxide nanoparticles. The cytotoxicity of MSA- or DMSA-coated magnetic nanoparticles (MNP) (thiolated nanoparticles) and nitrosated MSA- or nitrosated DMSA-coated MNPs (NO-releasing nanoparticles) were evaluated towards human lymphocytes. The results showed that MNP-MSA and MNP-DMSA have low cytotoxicity effects. On the other hand, NO-releasing MNPs were found to increase apoptosis and cell death compared to free NO-nanoparticles. Therefore, the cytotoxicity effects observed for NO-releasing MNPs may result in important biomedical applications, such as the treatment of tumors cells.

1. Introduction

The increasing exploration of nanotechnology in biological and medical applications has led to significant improvements in diagnosis, prevention, and treatment of diseases. Iron oxide magnetic nanoparticles (MNPs) have been explored in biomedical applications such as drug delivery [1-3] and therapy [4-6], since they are considered less toxic than their metallic counterparts. Moreover, MNPs offer further advantages such as high saturation magnetization and superparamagnetic behaviour. Among iron oxide MNPs, magnetite is a very promising choice due to its already proven biocompatibility.

Co-precipitation of Fe²⁺ and Fe³⁺ ions under alkaline conditions has been routinely used for the synthesis of magnetite nanoparticles (Fe₃O₄) [7], although more well-designed procedures involving
high temperature decomposition of iron(0) or iron(III) precursors stabilized by surfactant molecules have been reported [8]. The use of Fe₂O₃ and Fe₃O₄ nanoscale particles also includes drug targeting of cancer cells, tracking target cells using labeling, and imaging techniques like magnetic resonance tomography. In vivo studies with Fe₃O₄ nanoscale particles have demonstrated severe inflammatory and toxicity responses in rats exposed to nanoscale particles through inhalation [9-10]. Other studies have demonstrated that MNPs (Fe₃O₄) cause oxidative trauma to human bronchoalveolar epithelial and murine neuronal cells leading to loss of cell viability, and causing a change in electrical activity [11-12]. In contrast to these findings, other studies performed with microscale and nanoparticulate Fe₃O₄ described them both to be nontoxic under in vitro test conditions in human small airway epithelial and mouse fibroblast cells [13]. Therefore, it is of interest to observe the difference in the bio-nano interaction between Fe₃O₄ nano- and microscale particles in human lung cells [14] in vitro and to correlate the effect with their physicochemical properties.

Most studies under in vitro conditions consider the basic physicochemical characteristics of the nanoscale particles, such as shape, size, and surface coating. However, recent studies have shown that the surface of the nanoscale particles changes after interaction with the surrounding environment through solvation, protein corona formation, and agglomeration [15-16]. The biomedical applicability of MNPs is greatly improved by coating their surfaces with biocompatible ligands [17]. MNPs having suitable surface characteristics have a great potential for in vitro and in vivo applications. The effects of MNPs coated with biocompatible ligands containing functional groups such as COOH and SH as well as different sizes of MNPs are critical determinants in toxicological studies [18]. In this context, a good candidate to interact on the surface of these nanoparticles covered with biocompatible ligands is the nitric oxide (NO) molecule.

NO is involved in several physiological and pathophysiological processes, such as the control of vascular tone, the inhibition of platelet aggregation, and the immune response against microbes [19]. Thus, there is a great interest in the development of NO-releasing vehicles that are able to stabilize and release NO locally direct to the target site, in diverse biomedical applications.

This work reports the synthesis of surface-modified MNPs as vehicles to carry and deliver NO molecules and the evaluation of the cytotoxicity of these nanoparticles. MNPs, synthesised through the co-precipitation technique [7], were functionalized with NO. To this end, MNPs were coated with two thiol-containing ligands: mercaptosuccinic acid (MSA) and 2,3-dimercaptosuccinic acid (DMSA), leading to the formation of stable aqueous dispersions of thiolated nanoparticles (SH-MNPs). Free thiol (SH) groups were used as sites to covalently bind NO to the surface of MNPs. Cytotoxicity of SH-MNPs and NO-releasing MNPs were evaluated towards human lymphocytes. The results show that SH-MNPs were not cytotoxic. However, the presence of NO on the MNPs surface was responsible to increase cell death, apoptosis and cytotoxic effects, in a concentration-dependence manner. These results indicate that NO-releasing nanoparticles may result in important biomedical applications, such as the treatment of tumors.

2. Methods

2.1 Synthesis of MNPs
MNPs were synthesized by using the co-precipitation method, as previously reported [20, 21]. In brief, 4.0 mL of FeCl₃•6H₂O and 1.0 mL of FeCl₂•4H₂O (molar ratio 2:1), prepared in 1.0 mol/L HCl, were mixed and stirred while a volume of 50 mL of NH₄OH (0.7 mol/L) was added as precipitator. At this stage, the solution was centrifuged and the precipitate was decanted, followed by the addition of 6.0 mL of oleic acid. This mixture was then stirred for 20 minutes. The solution was centrifuged several times and the new precipitate was washed several times with ethanol and acetone, leading to MNP covered with oleic acid.
2.2 Adsorption of thiolated ligands
Oleic acid coated-MNPs (~10.0 mg) were then dissolved in 1.0 mL of toluene while MSA or DMSA (molar ratio Fe$_3$O$_4$:MSA or DMSA = 1:40) was dissolved in dimethyl sulfoxide (DMSO). 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-MNPs) [21].

2.3 Nitrosation of thiolated magnetic nanoparticles
Thiolated nanoparticles (SH-MNPs) were nitrosated leading to the formation of SNO-MNPs by adding excess of sodium nitrite solution [21]. Excess of unreacted nitrite was removed from MNP suspension by centrifugal ultrafiltration.

2.4 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 at a suitable location by a qualified professional, using disposable materials throughout the procedure. The lymphocytes were placed in RPMI 1600 culture medium (Cultilab) containing 300 µg/mL of L-glutamine and 200 µg/mL of NaHCO$_3$, 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% CO$_2$.

2.5 Incubation of cells with MNPs
Human lymphocytes cells were incubated with: SH-MNPs (both MSA- and DMSA-coated MNPs) and SNO-MNPs at nanoparticle concentrations (0.01; 0.05 and 0.1 mg/mL) for 1 h. In addition, the cells were also incubated with the nanoparticle ligands, (free MSA or free DMSA) at the same concentrations for 1 h. Negative and positive controls employed phosphate buffered saline (PBS) and H$_2$O$_2$ (200 µmol/L), respectively.

2.6 Tali analysis
Cell viability was measured using a Tali™ Apoptosis Kit consisting of Annexin V Alexa Fluor® 488 and Propidium Iodide (Invitrogen), and a Tali™ image-based cytometer, which enabled the numbers of viable, apoptotic, and dead cells to be counted. The cells that had been treated with SH-MNPs (both MSA- or DMSA-coated MNPs) and SNO-MNPs at nanoparticle concentrations (0.01; 0.05 and 0.1 mg/mL), nanoparticles were centrifuged and concentrated to 1 x 10$^6$ cells per mL. 100 µL aliquots of sample were prepared according to the specifications of the kit, and the tests were performed in triplicate.

3. Results and Discussion

3.1 Nitrosation of thiolated MNPs leading to nitrosated MNPs (SNO-MNPs)
Thiol groups on the surface of nanoparticles (SH-MNPs) were nitrosated by the addition of sodium nitrite, leading to the formation of SNO-MNPs. Indeed, nitrous acid is considered the nitrosating agent of SH groups leading to the formation of SNO groups [22]. Figure 1 shows schematically a representation of the coating of MNPs with MSA or DMSA (a), and subsequent functionalization of the nanoparticle surface with NO (b).
Figure 1. a) Ligand exchange of MNPs with oleic acid leading to the formation of SH-MNPs (coated either with MSA or DMSA; b) Functionalization of SH-MNPs with NO molecules [21].

### 3.2 Tali analysis – Cell viability

Overall, cell viability studies show that SH-MNPs were not toxic towards human fibroblasts (Figure 2). A detailed observation of the results indicates that MSA-MNPs can be considered to have slightly higher cell viability, compared to DMSA-MNPs. These results are consistent with published work, in which little cytotoxicity was observed upon incubation of fibroblasts with DMSA-MNPs [23]. In addition, exposure of lymphocytes to free MSA or DMSA (used as MNPs ligands) showed no toxic effects on cells. Although preliminary, these promising results indicate that thiolated MNPs (coated with either MSA or DMSA) can be used as an inert vehicle in drug delivery aimed at biomedical applications.

On the other hand, nitrosated nanoparticles (SNO-MNPs) decrease cell viability in comparison with the thiolated MNPs, for all tested concentrations (Figure 2). Thus, NO-releasing MNPs can be used to promote cytotoxicity.

### 3.3 Apoptosis and cell death

Figure 3 shows apoptosis and cell death upon incubation of human lymphocytes with thiolated MNPs (MSA- or DMSA-MNPs) and nitrosated MNPs (NO-releasing MNPs). It can be observed that NO-releasing MNPs caused higher apoptosis indices and cell death, compared with thiolated nanoparticles. Moreover, for NO-releasing MNPs, apoptotic cells indices were found to be higher in comparison with cell death. This preliminary result suggests that NO-releasing MNPs may have genotoxic effects on cell, under the experimental conditions.
Figure 2. Cell viability of human lymphocytes treated with thiolated MNPs (MSA-MNPs or DMSA-MNPs), and NO-releasing MNPs (SNO-MSA-MNPs or SNO-DMSA-MNPs), at different concentrations, as indicated in the Figure. Incubation time: 1 h. MNPs were also incubated with only the ligands: MSA or DMSA, under the same experimental conditions.

Figure 3. Percentage of apoptosis and cell death of human lymphocytes incubated with: thiolated MNPs coated with MSA (MSA-MNP) or DMSA (DMSA-MNP), nitrosated MNPs (SNO-MSA-MNP and SNO-DMSA-MNP), at different concentrations, as indicated in the Figure. Incubation time: 1 h. Cells were also incubated only with the ligands: MSA or DMSA, under the same experimental conditions.

Taken together, the results show a low value of apoptosis and cell death upon exposure of lymphocytes to MNPs. This result is in accordance with published work that reported a lower susceptibility of lymphocytes in comparison with other cell types [16].
On the other hand, these initial results show that NO-releasing MNPs increased cell apoptosis and death.

4. Conclusions

This work describes the preparation of MNPs as a new NO-delivery platform. Firstly, NPs were prepared through the co-precipitation method, and coated with thiol groups, leading to the formation of a stable aqueous dispersion of thiolated nanoparticles (SH-MNPs). Free thiols groups on the MNPs surfaces were used as sites to covalently bind NO. Toxicological evaluations of thiolated and NO-releasing MNPs were investigated using human lymphocytes. The results showed that thiolated MNPs (MSA- or DMSA-coated MNPs) were not cytotoxic, and did not cause apoptosis and cell death, indicating that these nanoparticles can be safely used as drug delivery vehicles. On the other hand, NO-releasing MNPs were found to cause toxic effects on cells, and to increase apoptosis and cell death. In this regard, NO-releasing MNPs might be considered a promising approach to combat tumors, since the nanoparticles can be guided to the target site through the application of an external magnetic field, and release NO directly to the desired tissue/cell, where NO can have its cytotoxic effect.

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6. References

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