Synthesis, Characterization and Cytotoxicity Evaluation of Nitric Oxide-Iron Oxide magnetic Nanoparticles

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Abstract. The present work is focused on the synthesis, characterization and cytotoxic evaluation of superparamagnetic iron oxide nanoparticles (SPIONs). SPIONs have been proposed for an increasing number of biomedical applications, such as drug-delivery. To this end, toxicological studies of their potential effects in biological systems must be better evaluated. The aim of this study was to examine the *in vitro* cytotoxicity of thiolated (SH) and S-nitrosated (S-NO) SPIONs in cancer cell lines. SPIONs were prepared by the co-precipitation method using ferrous and ferric chlorides in aqueous solution. The nanoparticles (Fe_3O_4) were coated with thiol containing molecule cysteine (Cys) (molar ratio SPIONs:ligand = 1:20), leading to the formation of an aqueous dispersion of thiolated nanoparticles (SH-SPIONs). These particles were characterized by Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), transmission electron microscopy (TEM) and vibrating sample magnetometer (VSM). The results obtnained showed that Cys-SPIONs have a mean diameter of 14 nm at solid state and present superparamagnetic behavior at room temperature. Thiol groups on the surface of the nanoparticles were nitrosated through the addition of sodium nitrite leading to the formation of S-NOCys-SPIONs (S-nitrosated-Cys-SPIONs, which act as spontaneous nitric oxide (NO) donor). The cytotoxicity of thiolated and S-nitrosated nanoparticles was evaluated in acute T cell leukemia (Jurkat cell line) and Lewis lung carcinoma (3LL) cells. The results showed that at low concentrations thiolated (Cys) and S-nitrosated (S-NOCyst) SPIONs display low cytotoxicity in both cell types. However, at higher concentrations, Cys-SPIONs exhibited cytotoxic effects, whereas S-NOCys-SPIONs protected them, and also promoted cell proliferation.

1. Introduction

Nanoparticles represent an attractive class of materials, which due to their small size exhibit novel properties that differ from bulk materials [1-2]. Of all the nanomaterials, superparamagnetic iron oxide nanoparticles (SPIONs) are of enormous interest due to their exclusive properties such as superparamagnetism, and biodegradability [3]. SPIONs have found applications in targeted drug delivery, magnetic resonance image (MRI) contrast enhancement, cancer diagnosis, hyperthermic treatment of tumors, magnetically mediated separation of biomolecules and waste water treatment [4-6]. In this context, iron oxide particles, such as magnetite (Fe_3O_4), are the most common investigated in biomedical applications [7-8]. In general, *in vitro* studies of SPIONs demonstrated little cytoxicity indicating the biocompatibility of this nanostructured systems [9-11]. Functionalization permits the adsorption of molecules of interest, such as biomolecules, giving a stable aqueous dispersion. Further, conjugation of SPIONs with biomolecules, in many cases, would provide special properties to the nanoparticles, such as drug delivery [12]. In this context, molecules containing thiol groups such as cysteine (Cys) can be nitrosated, leading to the formation of S-nitroso-Cys-SPIONs. The formation of S-nitroso-groups (S-nitrosothiols) on the surface of SPIONs leads to nitric oxide (NO)-releasing iron oxide magnetic nanoparticles, as previously reported by our research group [7-11]. 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 [13]. Thus, there is a great interest in the investigation 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 studies *in vitro* of the cytotoxicity of thiolated (SH) and S-nitrosated (S-NO) SPIONs in cancer cell lines. SPIONs were prepared by the co-precipitation method using ferrous and ferric chlorides in aqueous solution. The nanoparticles were coated with thiol containing molecule Cys (molar ratio SPIONs:ligand = 1:20), giving thiolated nanoparticles (Cys-SPIONs). Further Cys-SPIONs were nitrosated with an excess of an aqueous solution of sodium nitrite ($NaNO_2$) at pH= 6. These particles were characterized by Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), transmission electron microscopy (TEM) and vibrating sample magnetometer (VSM). The cytotoxicity of thiolated and S-nitrosated nanoparticles was evaluated in acute T cell leukemia (Jurkat cell line) and Lewis lung carcinoma (3LL) cells.

2. Methods

2.1. Synthesis of Cys-SPIONs

SPIONs were synthesized by using a co-precipitation method, as previously reported [10]. In brief, 4.0 mL of $FeCl_3 \cdot 6H_2O$ and 1.0 mL of $FeCl_2 \cdot 4H_2O$ (molar ratio 2:1), prepared 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. At this stage, the black suspension formed was decanted magnetically followed by the addition of Cys in molar ratio Fe_3O_4: ligand 1:20. This mixture was then stirred for 1 hour. The dispersion was centrifuged and the new precipitate was washed several times with ethanol leading to a SPIONs covered with Cys.

2.1. Determination of the amount of free thiol groups on the surface of SPIONs

The amount of free thiol groups (–SH) on the surface of the nanoparticles was measured by the 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) reaction, based on the absorbance at 412 nm (ε = 14,150 $molL^{-1}cm^{-1}$) of the 2-nitro-5-thiobenzoate anion (TNB^{2-}) generated in the reaction of –SH groups with DTNB [15]. Cys-SPIONs were added to 3.0 mL of DTNB (0.01 $molL^{-1}$) in PBS buffer (pH 7.4) with 1 $mmolL^{-1}$ of ethylenediaminetetraacetic acid. After 5 min of incubation, the suspensions were filtered by centrifugal ultrafiltration using a Microcon centrifugal filter device containing ultrafiltration

membranes (MWCO 10-kDa molar mass cut-off filter, Millipore, Billerica, MA, USA). The supernatant was placed into a quartz cuvette, and the intensity of the absorption band at 412 nm was measured in an Uv-Vis Spectrophotometer (Agilent, model 8553, Palo Alto, CA, USA). The experiments were carried out in triplicates.

2.2. Preparation of S-nitrosated SPIONs

Cysteine nanoparticles (Cys-SPIONs) were nitrosated leading to the formation S-nitroso-Cys-SPIONs by adding excess of $NaNO_2$ at slight acid pH [14]. Excess of unreacted nitrite was removed from SPIONs suspension by centrifugal ultrafiltration.

2.3. In vitro experiments

Cancer cell lines (K562, Lucena, Jukart and 3LL cells) were treated with different concentrations of Cys-SPIONs and S-nitroso-Cys-SPIONs for 24 h. Cytotoxicity assays were performed by tetrazolin reduction MTT (3-(4-5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide).

3. Results and Discussion

The amounts of free thiol (-SH) groups on the surface of Cys-SPIONs were determined by the reaction of the thiolated nanoparticles with a thiol-specific reagent, DTNB [15]. The quantity of 12 ± 8 μmol SH per gram of Cys-SPIONs was found, indicating a high extend of functionalization of SPIONs with Cys. Moreover, Cys-SPIONs were investigated by our research group by several physicochemical techniques, such as, FTIR, X-ray diffractometric, TEM and the hysteresis curves (data no shown). Therefore, the combined results obtained by all these techniques gave support to deduce that the covering of cysteine onto nanoparticles surface produced crystalline cysteine magnetite (Cys-Fe_3O_4) with spherical morphologies and narrow sizes distribution. Superparamagnetic behavior at room temperature was also observed.

Figure 1 shows the schematic representation the nitrosation of free thiol groups (SH) adsorbed on Fe_3O_4 surface (Cys-Fe_3O_4) by the addition of $NaNO_2$ in aqueous solution at pH slight acid, leading to the formation of S-nitroso-Cys-Fe_3O_4.

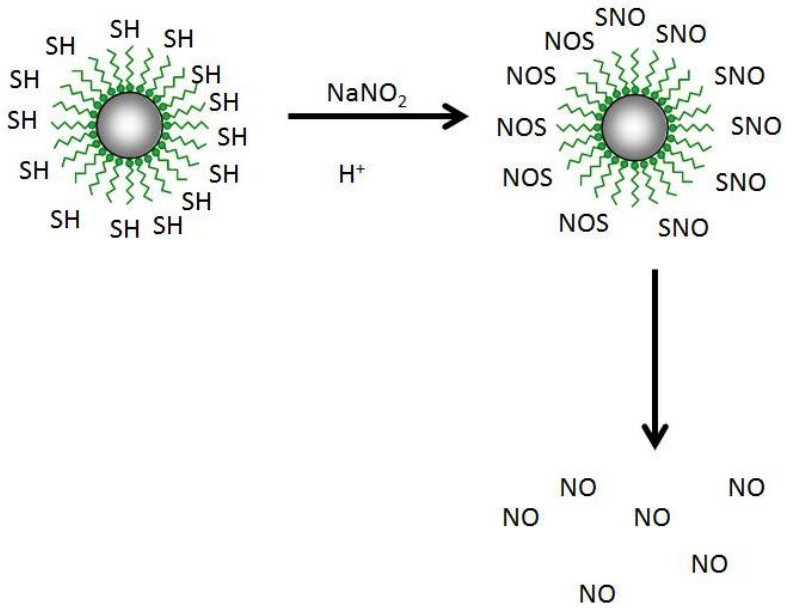

Figure 1. Schematic representation of the functionalization of SH-SPIONs with NO molecules.

S-nitroso groups (S-NO) act as spontaneous NO donor due to the homolytic S-N cleavage with free NO release (Fig. 1). Free NO released from the surface of SPIONs may have several effects in biological system. In order to propose the use of SPIONs in biomedical application, it is important to evaluate the cytotoxicity of the NPs. In this context, the *in vitro* toxicities of thiolated (Cys-SPIONs) and S-nitrosated (S-nitroso-Cys-SPIONs) nanoparticles were evaluated in acute T cell leukemia (Jurkat cell line) and Lewis lung carcinoma (3LL) cells.

Fig. 2 shows the cell viability of K562 cells after incubation with Cys-Fe_3O_4 and S-nitroso-Cys-Fe_3O_4 nanoparticles. It can be observed that both Cys-Fe_3O_4 and S-nitroso-Cys-Fe_3O_4 nanoparticles did not significantly decrease the cell viability at concentrations from 0.0001 to 0.01 mg/mL, when compared to the control cells. However, at nanoparticle concentrations of 0.25 and 0.5 mg/mL, thiolated nanoparticles (Cys-SPIONs) slight decrease cell viability. In contrast, S-nitrosated nanoparticles did no cause toxic effects, even at higher concentrations.

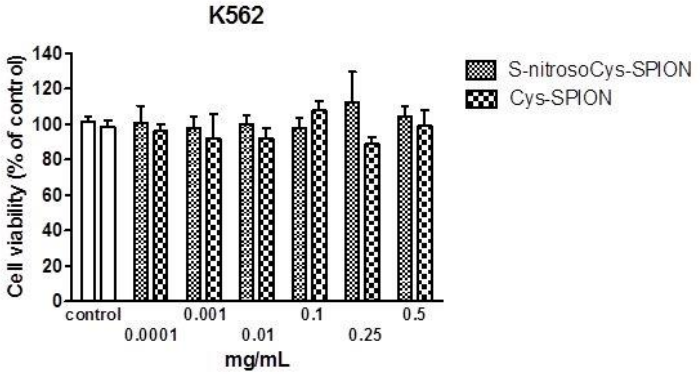

Figure 2. Cytotoxicity of Cys-SPIONs and S-nitroso-Cys-SPIONs in K562 cells. Chronic myeloid leukemia (K562) cells (1x105/mL) were treated with different concentrations of Cys-SPIONs and S-nitroso-Cys-SPIONs for 24 h, and afterwards, cell viability was analyzed by the resazurin reduction assay. The results represent means ± SD of one experiment run in sextuplicate. Data were expressed relative to control cell viability (100%).

Fig. 3 represents the viability cell of Lucena cells. Similarly, it was observed that nanoparticles did not reduce the cells viability at the lowest tested concentrations. By increasing the nanoparticles concentrations (0.1 up to 0.5 mg/mL), a discrete increase in cell viability upon cell incubation with S-nitroso-Cys-SPIONs could be noted, while a slight decrease in cell viability upon cell incubation with Cys-SPIONs could be observed.

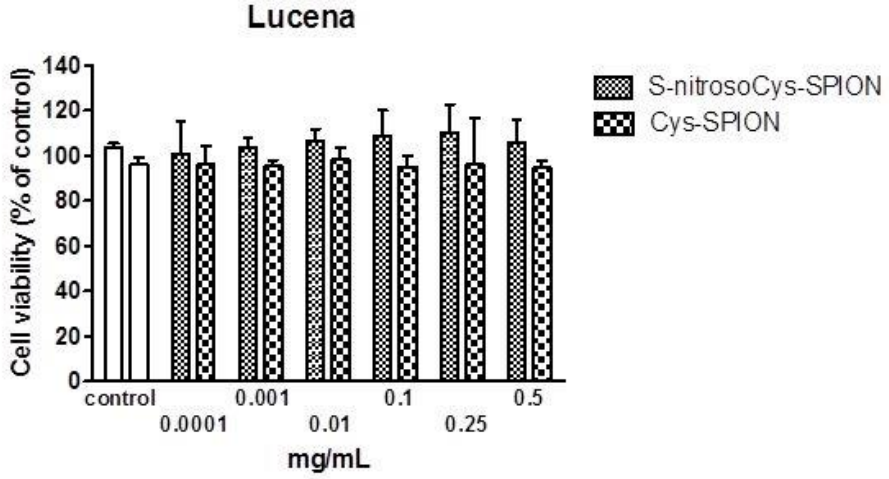

Figure 3. Cytotoxicity of Cys-SPIONs and S-nitroso-Cys-SPIONs in Lucena cells. Multidrug resistant chronic myeloid leukemia (Lucena) cells ($1x10^5$/mL) were treated with different concentrations of Cys-SPIONs and S-nitroso-Cys-SPIONs for 24 h, and afterwards, cell viability was analyzed by the resazurin reduction assay. The results represent means ± SD of one experiment run in sextuplicate. Data were expressed relative to control cell viability (100%).

Interesting results are shown for the essays using Jurkat and 3LL cells (Figs. 4 and 5, respectively). These cancer cells are more resistant, and a more evident cytotoxic effects of Cys-SPIONs could be observed, whereas S-nitroso-Cys-SPIONs protected the cells, and also promoted cell proliferation.

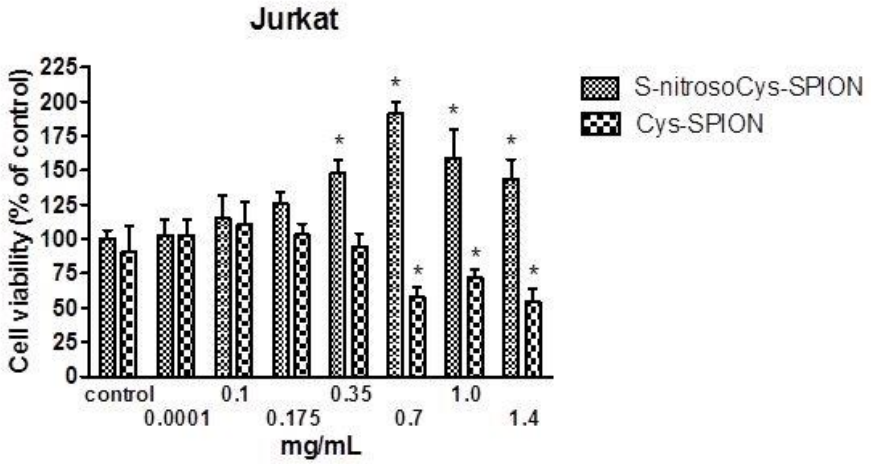

Figure 4. Cytotoxicity of Cys-SPIONs and S-nitroso-Cys-SPIONs in Jurkat cells. Jurkat cells ($1x10^5$/mL) were treated with different concentrations of Cys-SPIONs and S-nitroso-Cys-SPIONs for 24 h, and afterwards, cell viability was analyzed by the resazurin reduction assay. The results represent means ± SD of one experiment run in sextuplicate. Data were expressed relative to control cell viability (100%). *$p<0.05$ compared with control cells.

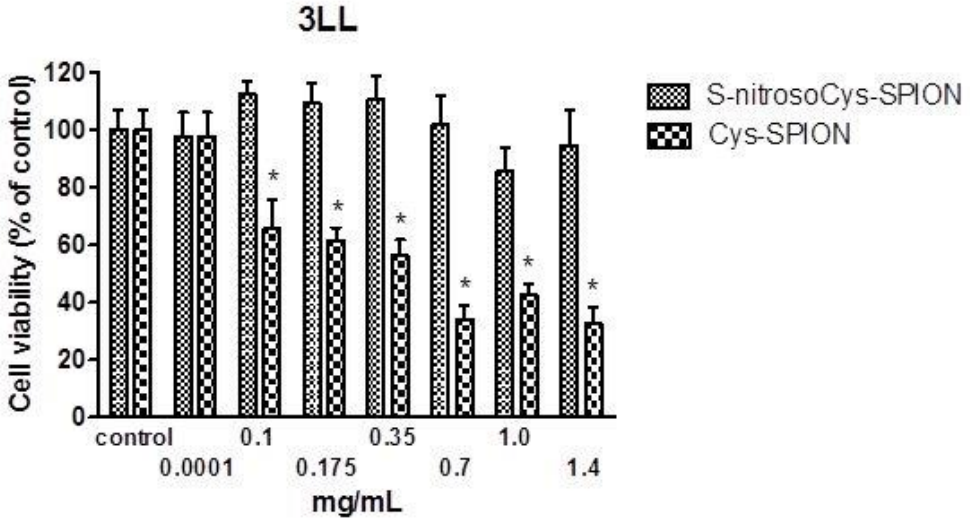

Figure 5. Cytotoxicity of Cys-SPIONs and S-nitroso-Cys-SPIONs in 3LL cells. 3LL cells ($1x10^4$/well) were treated with different concentrations of Cys-SPIONs and S-nitroso-Cys-SPIONs for 24 h, and afterwards, cell viability was analyzed by the resazurin reduction assay. The results represent means ± SD of one experiment run in sextuplicate. Data were expressed relative to control cell viability (100%). *$p<0.05$ compared with control.

Taken together, the preliminary results indicate the toxicity of Cys-SPIONs and S-nitroso-Cys is dependent on the cell type. In the case of K562 and Lucena cell lines, a significant decrease in cell viability was not observed for both nanoparticles at all tested concentrations. However, for Jurkat and 3LL cell lines, thiolated SPIONs were found to be toxic at higher tested concentrations, whereas S-nitrosated-SPIONs rescue cell toxicity. This interesting effect can be assigned to the complex biochemistry of NO, which is involved in several mechanism pathways in the biological system. Moreover, NO is reported to have a dichotomous effect on cancer cell, acting either to promote cell proliferation or to kill cancer cells. Further studies are required to better understand the mechanism involved.

4. Conclusions

SPIONs functionalized with Cys were successfully synthesized. Free thiol groups on the surface of the nanoparticles were nitrosated through the addition of sodium nitrite leading to the formation of S-nitroso-Cys-SPIONs. The cytotoxicity results showed that at low concentrations Cyst-SPIONs and S-nitroso-Cys-SPIONs exhibited low cytotoxicity in all cell types investigated. However, at higher concentrations, Cys-SPIONs exhibited cytotoxic effects, whereas S-nitroso-Cys-SPIONs protected them, and also promoted cell proliferation, in the case of Jurkat and 3LL cell lines. Further studies are undertaken to better investigate this effect.

5. Acknowledgements

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6. References

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