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Synthesis of iron oxide nanoparticles stabilized with sodium citrate and TMAOH

Síntese de nanopartículas de óxido de ferro estabilizadas com citrato de sódio e TMAOH

Síntesis de nanopartículas de óxido de hierro estabilizadas con citrato de sódio y TMAOH

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Abstract
Iron oxide nanoparticles (IONPs) represents a class of magnetic and biocompatible nanomaterials that have been widely used in research and medical applications, such as hyperthermia studies, as contrast agents for magnetic resonance imaging, biosensors, among others. However, their application depends on factors as surface properties, size, and morphology being appropriately balanced. IONPs can be obtained by different synthesis methods, however, chemical coprecipitation represents a simpler, easier and faster route, in which aqueous solutions of precursors containing iron (Fe^{3+}) and ferrous (Fe^{2+}) ions are alkalized under control of temperature and pH. This study proposes to synthesize iron oxide nanoparticles by the chemical coprecipitation method and to stabilize them with sodium citrate (IONPs-CIT) and tetramethylammonium hydroxide (IONPs-TMAOH). Furthermore, to characterize the hydrodynamic diameter and the Zeta Potential of the samples by Dynamic Light Scattering. The cytotoxicity of IONPs- CIT in the MDA-MB-468 cell line was evaluated through the analysis of mitochondrial activity.
Keywords: Iron oxide nanoparticles; Chemical coprecipitation; Dynamic light scattering; Cytotoxicity.

Resumo
As nanopartículas de óxido de ferro (IONPs) representam uma classe de nanomateriais magnéticos e biocompatíveis que têm sido amplamente utilizados em pesquisas e aplicações médicas, como estudos de hipertermia, como agentes de contraste para ressonância magnética, biossensores, entre outros. Entretanto, sua aplicação depende de fatores como, morfologia, tamanho e propriedade de superfície estejam ajustados. As IONPs podem ser obtidas por diferentes métodos de síntese, no entanto, a coprecipitação química representa uma rota de síntese mais simples, fácil e rápida, na qual soluções aquosas de precursores contendo íons ferro (Fe^{3+}) e ferroso (Fe^{2+}) são alcalinizadas sob controle de temperatura e pH. Este estudo propõe sintetizar nanopartículas de óxido de ferro pelo método de coprecipitação química e estabilizá-las com citrato de sódio (IONPs-CIT) e hidróxido de tetrametilamônio (IONPs-TMAOH). Além disso, caracterizar o diâmetro hidrodinâmico e o Potencial Zeta das amostras por espalhamento dinâmico da luz. A citotoxicidade das IONPs-CIT na linhagem celular MDA-MB-468 foi avaliada através da análise da atividade mitocondrial.
Palavras-chave: Nanopartículas de óxido de ferro; Coprecipitação química; Espalhamento dinâmico da luz; Citotoxicidade.

Resumen
Las nanopartículas de óxido de hierro (IONPs) representan una clase de nanomateriales magnéticos y biocompatibles que han sido ampliamente utilizados en investigación y aplicaciones médicas, tales como estudios de hipertermia, agentes de contraste para resonancia magnética, biosensores, entre otros. Sin embargo, su aplicación depende de un balance apropiado en factores como la morfología, el tamaño y las propiedades superficiales. Los IONP se pueden

obtener por diferentes métodos de síntesis, sin embargo, la coprecipitación química representa una ruta más simple, fácil y rápida, en la cual se alcalinizan soluciones acuosas de precursores que contienen iones de hierro (Fe^{3+}) y ferroso (Fe^{2+}) bajo control de la temperatura y el pH. Este estudio propone sintetizar nanopartículas de óxido de hierro por el método de coprecipitación química y estabilizarlas con citrato de sodio (IONPs-CIT) e hidróxido de tetrametilamonio (IONPs-TMAOH). Además, caracterizar el diámetro hidrodinámico y el Potencial Zeta de las síntesis de Dispersión Dinámica de Luz. La citotoxicidad de IONPs-CIT en la línea celular MDA-MB-468 se evaluó mediante el análisis de la actividad mitocondrial.
Palabras clave: Nanopartículas de óxido de hierro; Coprecipitación química; Dispersión de luz dinámica; Citotoxicidad.

1. Introduction

Nanoscience and nanotechnology are subjects widely discussed in scientific publications, which point out many potential uses of nanomaterials (structures with at least one dimension in the nanoscale, 1 to 100 nm). The reason is due to the possibility of manipulate materials organization in such a small scale that leads to the change of properties, when compared to the bulk (Mansoori, 2005). To state, the physical properties of gold nanoparticles has open opportunity for studies exploring their radiotherapy effects (Gialucca et al., 2021), production of nanocatalysts has been investigated for chemical reactions requiring oxidation and hydrogenation (da Silva Souto et al., 2022), liposomes, polymeric and lipid nanoparticles has been studied as nanocarriers for the pharmaceutical industry (Bezerra et al., 2022).

Nanoparticles can be obtained by the physical method with the "top-down" technique, which involves breakage of the material by wear and results in a wide granulometric and morphological distribution. Among the main approaches of this method, ion sputtering, ball milling, laser ablation and pyrolysis. In contrast, the "bottom-up" approach comprises the combination of atoms or molecules to form larger nanoparticles. The latter is used to produce more homogeneous nanoparticles with uniform size and shape distribution. This path encompasses chemical methods, such as Coprecipitation and Thermal Decomposition, and biological methods, with the application of different plants and microorganisms (Hernández-Hernández et al., 2020; Ansari et al., 2022; Niculescu, et al., 2021; Mokhosi et al., 2022; Gawel, et al., 2022; Bommakanti et al., 2022).

Iron oxide nanoparticles (IONPs) can be prepared by physical, chemical or biological processes. The physical method is characterized by easy development, however, one is unable to control the morphology and diameter. The chemical routes correspond to more simple and effective processes in which the morphology of the nanoparticles relies on the synthesis parameters, and can be spheres, platelets, bases, nanotubes, and nanorods, among others. The biological approaches are cheaper, with good reproducibility and yield, but usually laborious and time-consuming (Crețu et al., 2021; Niculescu, et al., 2021). Different chemical methods for synthesizing magnetic nanoparticles have been studied, such as hydrothermal, coprecipitation, thermal decomposition, microemulsion, laser electrochemical deposition, solvothermal, sonochemical, chemical vapor position, microwave assisted and aerochemical pyrolysis (Zhu et al., 2018).

As the behavior of IONPs in the biomedical field is influenced by three variables, including morphology, size and surface characteristics, experimental parameters such as the presence of surfactants, Fe^{2+}/Fe^{3+} ratio, salt type, pH control, electrical force, nature of the alkaline agent (NaOH, Na_2CO_3, NH_4OH), stirring rate, nitrogen or argon flow in the system, and temperature or time of reaction are some of the factors that can affect the physical characteristics of nanoparticles and influence their biological properties, as the rate of blood circulation, cellular absorption, and biodistribution (Hernández-Hernández et al., 2020; Crețu et al., 2021; Amatya et al., 2021). The shape, size, crystal structure, chemical composition, and dispersibility of nanoparticles directly affects their physical and chemical properties, and consequently also the performance of such materials in a bunch of applications (Shabatina et al., 2020; Ajinkya et al., 2020; Zhu et al., 2018).

IONPs are mostly composed of magnetite (Fe_3O_4), maghemite (γ-Fe_2O_3), or hematite (α-Fe_2O_3) and has high saturation magnetization, low area-to-volume ratio, and minimal cytotoxicity (Cretu et al., 2021; Wu et al., 2021; Canaparo et

al. 2020; Ansari et al., 2022). Some applications involving these nanomaterials for biomedical purposes includes, but are not limited to, biosensors, magnetic contrast agents, purification of proteins, immunoassays, cell markers, tissue repair, photothermal therapy, drug administration to patients, and drug separation. In cancer research, IONPs has been explored for boosting the reactivity of medications in combination therapies or as agents that cause hyperthermia (Hernandez-Hernandez et al., 2020; Wu et al., 2021; Canaparo et al., 2020; Mokhosi et al., 2022; Zhu et al., 2018; Dadfar et al., 2019; Fuller et al., 2019; Ajinkya et al., 2020; Brennan et al., 2020).

Among the methods for producing IONPs, the chemical coprecipitation of the oxides represents one of the most usual routes, due to the scale-up ability and easy procedures. The nanoparticles are formed by the precursors Fe^{2+} and Fe^{3+} (molar ratio 1:2) present in salts, under the addition of an alkaline solution, in order to keep the pH between 8-9, which favors mainly the formation of magnetite, and under inert atmosphere (e.g. by using nitrogen gas). (Shabatina et al., 2020; Hedayatnasab et al., 2020; Radoń et al., 2017; Hernández-Hernández et al., 2020; Crețu et al., 2021). The chemical protocol applied for producing nanomaterials should be well-defined, considering that the nature of the salt solution, reaction temperature, pH, and the ionic strength of the medium are the key determinants of the size, shape, and composition of the nanoparticles (Hernández-Hernández et al., 2020).

Irrespective of the intended use of IONPs, it is crucial to maintain the chemical stability. Otherwise, these materials may degrade in biological medium or aggregate, which leads to size increase and loss of the properties inherent to the nanoscale. In order to reduce the surface energy that is high, IONPs naturally aggregate (Laurent et al., 2008). Therefore, for prevent magnetic dipole interaction by steric or electrostatic repulsion, molecules must be adsorbed at the surface of IONPs. One alternative for IONPs stabilization through electrostatic repulsion is the addition of certain salts (e.g. sodium citrate) or surfactants (e.g. tetramethylammonium hydroxide, TMAOH) (Rosensweig, 1985; Andrade et al., 2012).

Sodium citrate can activate the surface of nanoparticles for some reactions with carboxylate ions (–COO−), allowing strong coordination of water highly with the Fe atom present in the nanoparticles (Ali Dheyab, et al., 2021), providing better dispersion and stability in water (Nguyen, et al., 2018). Molecules of citric acid and citrate salts can also undergo dissociation and hydrolysis processes in aqueous solutions to reach equilibrium, according to Krukowski et al. (2017), but it is known that these processes depend on solute concentration, solution pH, and temperature (Krukowski; et al., 2017). The surface of IONPs contains hydroxyl groups (OH^-), and in presence of TMAOH the stabilization occurs because the cations $[(CH_3)_4N]^+$ of the surfactant is adsorbed by OH^-, resulting in the electrostatic repulsion. Further, the peptization with TMAOH promotes dispersibility and better control over nanoparticles crystallinity and size (Andrade et al., 2012).

In this context, the present article proposes to synthesize iron oxide nanoparticles by the chemical coprecipitation method and to stabilize with sodium citrate and tetramethylammonium hydroxide. Furthermore, to characterize the samples by Dynamic Light Scattering (DLS), in order to determine the hydrodynamic diameter and Zeta potential, and evaluate the influence of the stabilizers in controlling the size of the end products. For both samples, the cytotoxicity in the MDA-MB-468 cell line will be evaluated through the analysis of mitochondrial activity.

2. Methodology

The methodology was based on the scientific method that allows the analysis and investigation of the results, which are related to standard methods described in the literature (Pereira, A. S et al., 2018; Estrela C., 2018; Köche, J. C., 2011; Ludke, M. & Andre, M. E. D. A, 2013; Yin, R.K., 2015; Severino, A. J., 2018).

The reagents description used are iron (II) chloride tetrahydrate 99% ($FeCl_2.4H_2O$), iron (III) chloride hexahydrate 97% ($FeCl_3.6H_2O$), sodium citrate dihydrate ($Na_3C_6H_5O_7.2H_2O$) solution 25% w/v, tetramethylammonium hydroxide(TMAOH) solution 25% w/v, iron standard for ICP, hydroxylamine hydrochloride 99% ($NH_2OH.HCl$), sodium

acetate > 99% (CH_3COONa), 1,10- phenanthroline monohydrate > 99%, Ammonium hydroxide (NH_4OH) 29% v/v and nitric acid (HNO_3), Deionized water was used throughout the experiments.

2.1 Synthesis of IONPS stabilized with sodium citrate and TMAOH

The coprecipitation approach was used to create IONPs, as described by Massart (1981) (adapted). In ultrapure water that had previously been deoxygenated with nitrogen gas, iron chloride solutions II ($FeCl_2.4H_2O$) and III ($FeCl_3.6H_2O$) at 0.2 mol/L and 0.4 mol/L, respectively, were produced separately. Stirring was maintained throughout the synthesis at a temperature of 85 °C and a pH of 8 to 9. The precipitation of iron oxides occurred through the addition of 35 mL of ammonium hydroxide (NH_4OH) (Massart, R.,1981). The IONPs were stabilized according to Mérida et.al. (2015) (adapted) with sodium citrate dihydrate (0.25M) (IONPs-CIT.) and peptized with TMAOH (1M) (IONPs-TMAOH), in two different processes, and in both cases, the samples were submitted to 30 minutes of ultrasound (150kJ) in an ice bath. The process was repeated for greater stabilization of the nanoparticles, and final centrifugation at 2,500 rpm for 10 min was performed. The supernatants were discarded and the black pastes were dried in air overnight and stored at 4°C (Mérida, F. et al., 2015).

2.2 Iron quantification by UV absorption assay

Using UV-visible spectroscopy, the iron concentration present in the IONPs was identified. IONPs stabilized with sodium citrate dehydrate, as well as those peptized with TMAOH were digested in 70% nitric acid (HNO_3) for around 12 hours in a dry bath at 101°C. Subsequently, 10 µL of the digested sample was evaporated at 115°C, followed by the addition of 46 µL of deionized water. For the reduction of Fe^{3+} ions to Fe^{2+}, 30 µL of hydroxylamine hydrochloride (NH_2OH. HCl) was added and the reaction was kept at rest for 1 h. After reduction, 49 µL of sodium acetate (NaO_2CCH_3) was added as a buffering agent and 75 µL of 1,10-phenanthroline monohydrate ($C_{12}H_8N_2$. H_2O), which formed the Fe(II)-orthophenanthroline complex. The absorbance of that complex was measured on spectrophotometer, Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, USA) at a wavelength of 508 nm, which is a quantitative analysis.

2.3 Characterization of the hydrodynamic diameter and surface charge

The hydrodynamic size and Zeta potential were determined by Dynamic Light Scattering using the equipment ZetaSizer nano – ZS90 (Malvern), which is a qualitative analysis. A volume of 200 µL of nanoparticles was added to the polystyrene cuvette (model ZEN0118, Sarstedt) to verify the particle size distribution. Three measurements were taken at a measuring angle of 90°, in 10 runs and a duration length of 30 seconds each. Zeta Potential analysis determined the surface charge of the colloidal solutions, when an electric potential differential was applied through a cuvette with gold electrodes. Using a syringe, the solutions were injected into capillary cuvettes with electrodes (model DTS1070, Malvern). The mathematical model defined was by Smoluchowski, an average of three analyzes per sampling was performed to obtain the results.

2.4 In vitro cytotoxicity

The human mammary adenocarcinoma cell line, MDA-MB-468 (Rio de Janeiro cell bank, BCRJ), was cultivated in sterile 25 cm^2 polypropylene bottles with L-15 culture medium (Leibovitz, Sigma Life Science, L4386-10x1L) and supplemented with 10% (v/v) Fetal Bovine Serum (Life Technologies, 16000-044). The cell culture was kept at 37 °C with a 95% humidity level (Thermo Scientific, water Jacket series 8000). After trypsinization with 0.25% Trypsin-EDTA solution, the medium was changed every two days and the cells were subcultured every 15 days. All experiments with this cell line were performed in triplicate.

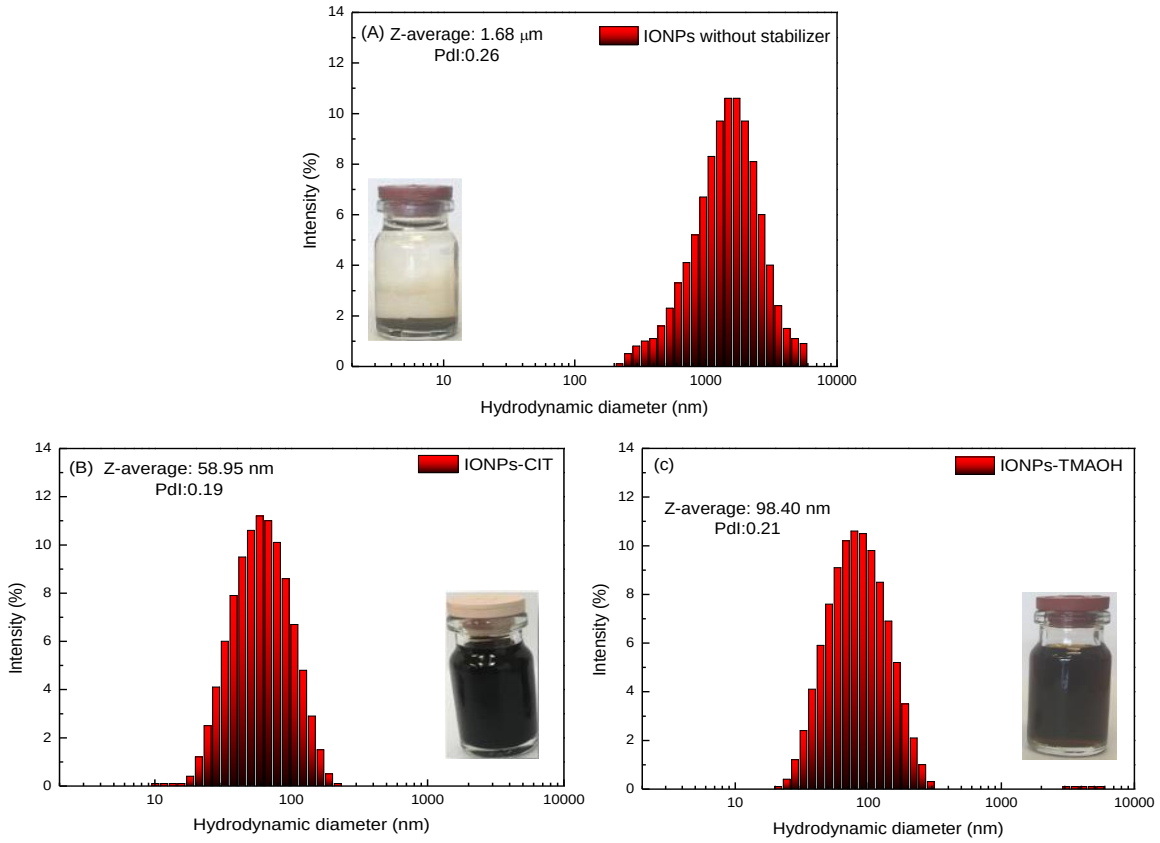
Viable cells in the MDA-MB-468 lineage were determined by the methodologies: colorimetric with MTT (3-(4,5-dimethyl)thiazol-2-yl-2,5-diphenyl tetrazolium bromide, Sigma Aldrich, M5655); and method of exclusion of use the dye trypan blue (3,3'-(3,3'-dimethyl[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino - 4-hydroxynaphthalene-2,7-disulfonate, Sigma Aldrich, T8154). The colorimetric procedure with MTT evaluated the mitochondrial activity, which was performed through the reduction of tetrazolium and formation of violet-colored Formazan crystals, with absorption at 570 nm (Stockert, 2012). The absorption reading was performed in triplicate using the Synergy HT Multi-Detection Microplate Reader spectrophotometer (BioTek Instruments, USA). Trypan blue color contrast contrasted the cell viability, with method comparison of nonviable and translucent cells as viable cells. By inverted microscopy NIB-100 (Bel Equipamentos Analíticos), 15 images were obtained by the Future WinJoe program, version 1.0.7.9. Cell counts were performed using the ImageJ program, version 1.48.

For the cytotoxicity assay, a $1x10^5$ cell suspension/mL (MDA-MB-468) was applied to 96-well plates containing a complete culture medium. After 24 hours of assessment, the medium was replaced by IONPs-CIT (5, 25, 50, 100, 150 and 300 µg/mL). The cells were incubated for 1, 2, 4 and 6 hours, under culture conditions. After the incorporation time, the medium containing colloidal solutions was removed, the wells were washed with PBS and a new complete culture medium was added and the plate was incubated for 20 h. After this period, trypan blue and/or MTT were added to the wells. The measurements results allow a quantitative analysis.

3. Results and Discussion

The IONPs had shown interaction with the applied external magnetic field (Neodymium magnet) and black color, that is attributed to the Fe_3O_4 phase. The hydrodynamic diameter of the IONPs without stabilizer, IONPs-CIT and IONPs-TMAOH was measured by DLS. The size distribution of the IONPs (without stabilizer, Figure 1A) suggested particles with 1.68 µm and the polydispersity index (PdI) 0.26, indicating the existence of aggregates and wide size distribution. However, IONPs stabilized with sodium citrate (IONPs-CIT) had a diameter of 58.95 nm and a PdI value of 0.19 (Figure 1B), which indicates that citrate ions enhance the control over the size distribution, maintain the material's dimensions at the nanoscale. The IONPs-TMAOH had a diameter of 98.40 nm and PdI 0.22 (Figure 1C). At pH 8, stabilized IONPs had a value of -48.6 mV, indicating the stabilization of these nanoparticles.

Figure 1 - Size distribution of IONPs (A) without stabilization, (B) with stabilization (IONPs-CIT) and (C) with stabilization (IONPs-TMAOH).



Source: Authors.

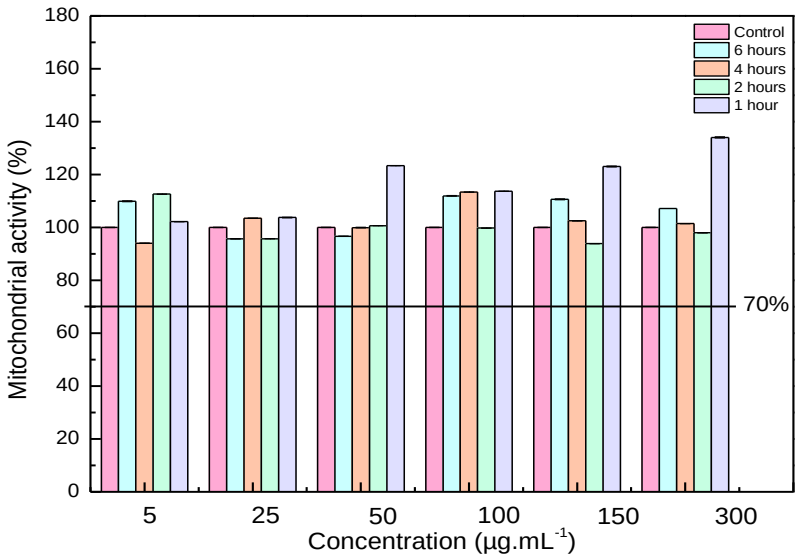
According to Cretu and coauthors (2021), as IONPs have a high area/volume ratio, magnetization, Van der Waals forces and high surface energy, they tend to aggregate (Crețu et al., 2021). Therefore, these nanoparticles can have their surface stabilized, coated and/or functionalized, which provides greater applicability (Amatya et al., 2021; Crețu et al., 2021). Both stabilization processes of the IONPs were efficient because a reduction in the hydrodynamic diameter was observed, when compared to the size of IONPs without stabilizer. Further, the colloidal stability of IONPs-CIT and IONPs-TMAOH was confirmed by Zeta Potential. These results are in agreement with Prokopiou et al. (2021) who synthesized iron oxide nanoparticles by the modified coprecipitation method using trisodium citrate, which through electrostatic interactions were a passive layer on the surface of the nanoparticles. In this study, the authors reported that the citrate-functionalized IONPs had a hydrodynamic diameter of 319±20.98 nm with PdI 0.55±0.09 and Zeta Potential of -41.1±0.46 mV (Prokopiou et al., 2021).

A crucial physical characteristic that affects the magnetic properties and surface area of IONPs is their diameter (Mokhosi et al., 2022), and information about it can be obtained through DLS or Transmission Electron Microscopy (TEM). Although complementary, they can reveal different sizes of IONPs. The TEM analysis provides the values of the physical diameter of the nanoparticles, allowing the determination of the presence or absence of aggregates, as well as morphological information, that is, it distinguishes the particles separately. Meanwhile, DLS provides an average hydrodynamic diameter through the intensity of the light scattered by the particles in Brownian motion (Rost et al., 2020). Usually the hydrodynamic size is larger than the physical size, because some molecules (e.g. organics) attached to the surface of nanomaterials are electron transparent (and therefore not imaged by TEM), but contributes to the light scattering when analyzed in DLS. The result of these analyses is directly influenced by the presence or absence of aggregates (Rost et al., 2020), molecules functionalized on the surface, and even by the magnetic interaction of particles (Youhannayee et al., 2019). In the case of

IONPs, a third kind of size is also important, the magnetic diameter, and this can be collected through magnetization curves. During the coprecipitation of iron oxides for forming nanoparticles, several chemical reactions takes place before produce magnetite. However, many others iron oxides phases can be present at the end of the synthesis, with a saturation magnetization lower than the observed for magnetite, forming a magnetically dead layer at the top of the magnetite core (Unni et al., 2017). Therefore, the synthesis and stabilization parameters are extremely important for standardizing the physical and chemical characteristics and applicability of IONPs (for example, for controlling the reactions pathways during iron oxides coprecipitation, which influences the magnetic diameter).

Figure 2 shows the cytotoxicity test of IONPs-CIT in the cell line MDA-MB-468 through mitochondrial activity. For this test, iron was initially quantified through the formation of the Fe(II)-orthophenanthroline complex, which has a maximum absorbance at 508 nm. The IONPs-CIT used to study the cytotoxicity, after quantification, presented Fe_3O_4 concentration equal to 2.36 mg. mL^{-1} and then IONPs-CIT solutions were prepared at concentrations of 5, 25, 50, 100, 150 and 300 µg/mL. The colorimetric method with MTT assess cell viability (Nepomuceno et al., 2021; Nandi et al., 2017), determining the minimum concentration necessary to obtain more than 50% viable or non-viable cells, with quantification of the activity of dehydrogenase from the reaction products NADH or NADPH, which reduces the activity of the MTT formazan crystals in living cells (Nepomuceno, et al., 2021).

Figure 2 - Analysis of MTT with IONPs-CIT in the MDA-MB-468 cell line.



Source: Authors.

According to the Standard (2009), ISO 10993-5: 2009 (E), it is established that the feasibility of cells can be determined through metabolic activity. If the feasibility is below 70%, it is understood that there is a cytotoxic potential (Standard, 2009). This results, inform that the nanoparticles did not show significant cytotoxicity, when in concentrations from 5 to 300 µg/mL, with an incubation time of up to 6 h. The biocompatibility and biosafety of the use of IONPs-CIT can also be confirmed in the findings of Prokopiou et al. (2021) who performed a cytotoxicity study of iron oxide nanoparticles functionalized with trisodium citrate in human keratinocyte cells, NCTC2544 and in a cell line with epithelial morphology isolated from the kidney of a human embryo, HEK-293. In this work, concentrations of 1,10,25,50,100 and 200 µg/mL were studied, observing high values of mitochondrial activity in the NCTC2544 line and reduction of viable cells in the HEK-293 lineage when in high concentrations. However, in both cell lines, all nanoparticles showed mitochondrial activity values above 70% (Prokopiou et al., 2021).

4. Conclusion

IONPs were successfully synthesized by the chemical coprecipitation method and stabilized with sodium citrate and TMAOH, which were confirmed by the DLS results, with particles smaller than 100 nm in diameter, stable and dispersed when in solution. The potential applicability in the use of these nanoparticles for biological purposes was suggested through the cytotoxicity test of IONPs-CIT through mitochondrial activity in the MDA-MB-468 cell line, which corresponds to triple-negative breast cancer, and has few treatment options and a worse prognosis.

The present study emphasizes the importance of the synthesis, parameters to obtain nanoparticles stable and dispersed, as well as, made possible suggestions for future research that include other characterization methods and biomedical analysis.

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