Synthesis, characterization and cytotoxicity of chitosan-coated Fe₃O₄ nanoparticles functionalized with ascorbic acid for biomedical applications

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Abstract. Iron oxide magnetic nanoparticles (Fe₃O₄ NPs) are used to drive and to promote sustained release of drugs in target sites. Biocompatibility and superparamagnetic behaviour are important features for the successful biomedical applications of Fe₃O₄ NPs. In this study, Fe₃O₄ NPs were synthesized by the co-precipitation method and coated with chitosan (CS) containing ascorbic acid (AA), allowing formation of Fe₃O₄@CS-AA NPs. The antioxidant AA was used as a drug model. The synthesized NPs were characterized by different techniques. The results showed the formation of spherical nanoparticles with average diameter of 67.22 ± 0.82 nm, at solid state, as analysed by atomic force microscopy (AFM). The NPs were found to have a superparamagnetic behaviour at room temperature, and the presence of CS-AA on the surface of Fe₃O₄ NPs did not affect the superparamagnetic behaviour of the nanoparticles. The in vitro AA release assay showed a sustained release of the model drug from Fe₃O₄@CS-AA NPs for at least 48 h. In addition, cytotoxicity assays for Fe₃O₄ NPs and Fe₃O₄@CS-AA NPs did not show significant toxicity towards mammary epithelium (MCF-10A) cell line after 24 h of incubation. This present study demonstrated the successful synthesis of superparamagnetic and biocompatible Fe₃O₄@CS-AA NPs, which are able to release the model drug in a sustained manner. Thus, this nanomaterial might act as a nanocarrier in target drug release.

1. Introduction

Nanotechnology is an interdisciplinary field that has been receiving a considerable attention in the last decades [1]. Nanotechnology has promoted scientific and technological advances in environment, energy and, particularly, in medicine [1]. Iron oxide nanoparticles (Fe₃O₄ NPs) hold great potential in several biomedical applications, such as targeted drug delivery, magnetic resonance imaging (MRI) and hyperthermia [2]. Fe₃O₄ NPs have a unique feature that is central to their application, the superparamagnetic behaviour at room temperature [2]. This magnetic phenomenon confers the ability to guide the nanoparticles to a specific organ or tissue by an external magnetic field or heat generation upon alternating magnetic fields [2]. The ability to specific target is desired in development of new approaches in drug delivery since it might potentiate drug effects while decreases side effects [3,4].

In this study, Fe₃O₄ NPs were synthesized and coated with chitosan (CS) containing ascorbic acid (AA), as drug model. Uncoated Fe₃O₄ NPs have some drawbacks, such as low dispersion in water, which can lead to particle aggregation and precipitation under physiological conditions [2]. Generally, Fe₃O₄ NPs are coated with biocompatible materials such as polymers, antibodies and peptide for biomedical applications [2,3,5]. This surface coating is important to avoid oxidation and agglomeration of the nanoparticles, increasing their biocompatibility [3,5].

CS is a biopolymer with intrinsic features such biocompatibility, biodegradability and antimicrobial activity [6]. It is a cationic polysaccharide composed of β-1,4-glucosamine monomers. CS is widely used in pharmacology applications and as coating material for metal oxide nanoparticles [6,7,8]. AA is a powerful antioxidant molecule [9] highly soluble in aqueous medium, biodegradable and non-toxic [10].
The purpose of this study was to describe the synthesis, characterization and cytotoxicity of superparamagnetic Fe₃O₄ NPs coated with CS containing AA. The morphological, size distribution and magnetic properties of the nanomaterial were evaluated, as well, the potential of Fe₃O₄ NPs to act as drug delivery system. In addition, the cytotoxicity of the synthesized nanoparticles was evaluated. The results suggest the ability of superparamagnetic and biocompatible Fe₃O₄ NPs in drug delivery.

2. Methods

2.1 Synthesis of Fe₃O₄ NPs and Fe₃O₄@CS NPs
Fe₃O₄ NPs were synthesized by the co-precipitation method [3,11,12,13]. First, aqueous solutions of iron II and III salts were prepared and mixed (1.57 mol L⁻¹ of FeCl₂·4H₂O and 0.84 mol L⁻¹ of FeCl₃·6H₂O) in acidified medium (HCl, 1.0 mol L⁻¹). Then, an aqueous solution of NH₄OH (0.7 mol L⁻¹) was dripped into mixed iron salts solution under vigorous magnetic stirring at room temperature. The addition of NH₄OH led to the formation of a black precipitate of Fe₃O₄ NPs. The obtained Fe₃O₄ NPs was washed, separated from the supernatant using a magnet, and freeze-dryer. CS-coated Fe₃O₄ NPs were prepared by mixing 0.2 g of Fe₃O₄ NPs with 0.1 g of CS, previously dissolved in 12 mL of acetic acid (0.175 mol L⁻¹), under stirring for 1 h at room temperature. The final suspension of Fe₃O₄@CS NPs was isolated, washed and dried.

2.2 Preparation of Fe₃O₄@CS-AA NPs
Firstly, Fe₃O₄ NPs were dispersed in 200 mL of ultra-purified water (1 mg mL⁻¹) followed by mixing with 12 mL of acetic acid solution (0.175 mol L⁻¹) containing 0.1 g of CS (30.2% w/w) and 0.03 g of AA (9.9% w/w), under vigorous magnetic stirring for 1 h at room temperature, yielding Fe₃O₄ NPs coated with CS and AA (Fe₃O₄@CS-AA NPs). The obtained Fe₃O₄@CS-AA NPs were washed, isolated and dried.

2.3 Dynamic light scattering (DLS) measurements
The hydrodynamic size, polydispersity index (PDI) and zeta potential of Fe₃O₄ NPs, Fe₃O₄@CS NPs and Fe₃O₄@CS-AA NPs were evaluated by DLS (Nano ZS Zetasizer, Malvern Instruments Co, UK) [4]. The nanoparticles were dispersed in ultra-purified water. Measurements were performed at 25°C in a 10 mm optical path disposable capillary cuvette (DTS1070).

2.4 Atomic force microscopy (AFM)
The morphology and size distribution at solid state of Fe₃O₄ NPs and Fe₃O₄@CS-AA NPs were evaluated by AFM. Nanoparticles were dispersed in ultra-purified water and dropped on a silica substrate. Solid state particles size distribution was measured by AFM (Agilent, AFM/STM Series 5500) using non-contact mode tip (Nanoworld, 320 kHz, 42 N m⁻¹). Images generated by the AFM were treated in the WSxM 5.0 Develop 8.2 and OriginPro 8 software. Particle distribution histograms were obtained by automatic particle counting of Fe₃O₄ NPs and Fe₃O₄@CS-AA NPs topographic images. It was carried out the analysis of three different topography representative images obtained for Fe₃O₄ NPs and for Fe₃O₄@CS-AA. It was employed the automatic counting of a total of 180 particles for Fe₃O₄ NPs and 22 particles for Fe₃O₄@CS-AA NPs by using the software WSxM 5.0 Develop 8.2 to obtain the distribution histograms for the nanoparticles [14].

2.5 Functionalization efficiency (FE%) of AA on the surface of Fe₃O₄@CS-AA NPs
Dispersion of Fe₃O₄@CS-AA NPs was prepared and filtered using a Microcon centrifugation filtration system (MWCO 10,000 kDa, Millipore), in order to separate free AA from AA presented on the surface of Fe₃O₄@CS NPs. The presence of free AA was quantity in the eluted sample by measuring the intensity of the absorption band of AA at 262 nm (ε = 10,216.0 Lmol⁻¹cm⁻¹) by using a UV-vis spectrophotometer (Agilent, model 8454) [15,16].

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2.6 X-ray diffraction (XRD)
The XRD assays for Fe₃O₄ NPs and Fe₃O₄@CS-AA NPs were performed using the X-ray diffractometer by STOE (STADI-P model) in a 20 scattering angular range from 2 to 79.985° at an integration time of 60s at each 0.78° using molybdenum source. The identification of nanoparticles crystalline structure was performed by comparison to diffractograms obtained from tabulated standards. The grain sizes of the Fe₃O₄ NPs and Fe₃O₄@CS-AA NPs were determined using the Debye-Scherrer equation [17,18].

2.7 Fourier transform infrared spectroscopy (FTIR)
Fe₃O₄ NPs and Fe₃O₄@CS-AA NPs were analyzed using an infrared spectrometer (Shimadzu, Prestige-21) at 2 cm⁻¹ resolution, in the range of 4000-400 cm⁻¹ in addition to the CS and AA control groups. Nanoparticles and control groups were pressed and analyzed on KBr pellets.

2.8 X-ray excited photoelectron spectroscopy (XPS)
XPS was used to verify the surface composition of Fe₃O₄ NPs. The equipment used was a spectrometer (ThermoFisher Scientific, model K-alpha+) with Al-K radiation. The analysis chamber pressure was kept in the range of 10⁻⁹-10⁻⁸ mbar and depth of reach is 10 nm. The contribution of each component was obtained from the deconvolution of the high-resolution spectra using a procedure based on peak adjustment by means of Gaussian-Lorentzian functions.

2.9 Superconducting quantum interference device (SQUID)
Magnetization measurements of Fe₃O₄ NPs and Fe₃O₄@CS-AA NPs were performed using a superconducting quantum interference device (SQUID) magnetometer (Quantum Design, VSM-SQUID) at 300 K varying magnetic field between -30 and 30 kOe. The measurements were performed on dried powder, which was slightly pressed and conditioned in cylindrical holders.

2.10 In vitro AA diffusion from Fe₃O₄@CS-AA NPs
The kinetics of AA diffusion from Fe₃O₄@CS-AA were performed using the Franz vertical diffusion cell (Standard Model, 15 mm, 7 mL, Hanson Research Corporation), and compared with the kinetics of free AA diffusion. The donor compartment was filled with 2.0 mL of (i) free AA solution (30 mg mL⁻¹) or (ii) aqueous suspension of Fe₃O₄@CS-AA NPs (200 mg mL⁻¹, which corresponds to 30 mg mL⁻¹ of AA). A hydrophilic artificial membrane (Merck Millipore, 0.45 μm pore size) was used to separate donor and receptor compartments. The receptor compartment was filled with 6.7 mL of PBS at 25°C under constant magnetic stirring (350 rpm). Aliquots of 500 μL were collected at time intervals from the recipient compartment (0 – 48 h) with media replacement. Samples were analyzed by UV-Vis spectroscopy (Agilent, model 8454), at 262 nm, for quantification of diffused AA from donor compartment to receptor compartment. The results were expressed as percentage of AA released for each group (n=3). The maximum amount of AA was calculated by integral molar concentration vs time curve [19].

2.11 Cytotoxicity of Fe₃O₄ NPs, Fe₃O₄@CS NPs and Fe₃O₄@CS-AA NPs
The cytotoxicity effects of Fe₃O₄ NPs, Fe₃O₄@CS NPs and Fe₃O₄@CS-AA NPs were evaluated towards human breast epithelium (MCF-10A, ATCC) cell line. Different concentrations of nanoparticles (10, 30, 50, 70, 100, 200, 300 or 400 μg mL⁻¹) were added to each well containing 2 x 10⁴ cells/cm² followed by incubation for 24 h, at 37°C, in a 5% CO₂ atmosphere. Then, the media was changed and 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg mL⁻¹) was added to each well followed by 45 min incubation with 150 μL of DMSO. The absorbance of samples at 570 nm were evaluated on a plate reader. The control was performed in the absence of nanoparticles and considered as 100% of cell viability. Each point represents the average of two independent experiments, with the error bar values expressed by their standard error of the mean (SEM).
3. Results and Discussion

3.1. Size distribution and morphological characterization of Fe₃O₄ NPs, Fe₃O₄@CS NPs and Fe₃O₄@CS-AA NPs

Fe₃O₄ NPs were synthesized by the co-precipitation of Fe²⁺ and Fe³⁺ salts, in the presence of NH₄OH. The surface of Fe₃O₄ NPs was coated with the biopolymer CS by the electrostatic interactions of hydroxyl groups (OH⁻) present on the surface of Fe₃O₄ NPs and protonated amine groups (NH₃⁺) of CS. The presence of CS stabilized Fe₃O₄ NPs and improved the AA anchoring on the surface of the nanoparticles.

DLS measurements showed hydrodynamic sizes of 287.63 ± 37.21, 77.19 ± 2.5 and 54.09 ± 0.47 nm for Fe₃O₄ NPs, Fe₃O₄@CS NPs and Fe₃O₄@CS-AA NPs, respectively. PDI values of 0.361 ± 0.014, 0.158 ± 0.002 and 0.276 ± 0.05 were found for Fe₃O₄ NPs, Fe₃O₄@CS NPs and Fe₃O₄@CS-AA NPs, respectively. These results are in accordance with literature, Seabra et al. showed a hydrodynamic size of 226 ± 19 nm and a PDI index of 0.30 ± 0.001 for Fe₃O₄ NPs coated with mercaptosuccinic acid (MSA) [4] and Santos et al. showed a hydrodynamic size of 155.5 ± 3.61 nm and a PDI index of 0.208 ± 0.004 for Fe₃O₄ NPs coated with PEG [3]. Ding et al. reported the synthesis Fe₃O₄ NPs coated with chemically modified CS with hydrodynamic size of 65-67 nm, similar to our results [20]. The coating of Fe₃O₄ NPs with CS and AA decreased hydrodynamic size and PDI because the coating is responsible to avoid particle aggregation. In contrast, uncoated Fe₃O₄ NPs demonstrated higher hydrodynamic size and PDI values probably due to a high degree of nanoparticles agglomeration and aggregation. The PDI values reported here indicate a moderate polydispersity.

The zeta potential values for Fe₃O₄ NPs, Fe₃O₄@CS NPs and Fe₃O₄@CS-AA NPs were -30.43 ± 3.8 mV, +40.7 ± 6.8 mV and -18.26 ± 0.98 mV, respectively, indicating good stability of all nanoparticles in aqueous medium. The positive value of zeta potential of Fe₃O₄@CS NPs confirms the presence of CS layers on the surface of uncoated Fe₃O₄ [19]. CS has a positive charge due to the presence of NH₃⁺ groups [6]. In contrast, the observed negative zeta potential value of Fe₃O₄@CS-AA NPs is due to the presence AA on the surface of the nanoparticles. Our zeta potential value for uncoated Fe₃O₄ NPs is similar to the value reported by Ding et al. [20]. The results are in accordance with literature, Zhu et al. showed the value of -13.40 mV for the zeta potential of uncoated Fe₃O₄ NPs, and +54.20 mV for CS coated Fe₃O₄ NPs [21]. Feng et al. showed a negative value of zeta potential of -17.0 mV for Fe₃O₄ NPs coated with AA [22].

AFM was used to characterize the morphology and size distribution at solid state of Fe₃O₄ and Fe₃O₄@CS-AA NPs (Figure 1). It can be observed that the nanoparticles have a spherical shape and are well dispersed. The mean diameter size distribution for Fe₃O₄ NPs and Fe₃O₄@CS-AA NPs were found to be 51.94 ± 1.33 and 67.22 ± 0.82 nm, respectively (Figure 1). Our results agree with published works that report size values for uncoated Fe₃O₄ NPs of 50 nm and for coated Fe₃O₄ NPs of 100 nm [20,23].

In addition, the presence of CS-AA on the surface of Fe₃O₄ NPs increased the mean size distribution. The functionalization efficiency (FE%) of AA on the surface of Fe₃O₄@CS-AA NPs was found to be 99.8 ± 0.01%. That indicates a high affinity of AA to Fe₃O₄@CS NPs. Alishahi et al. observed a strong electrostatic interaction of AA with CS and they showed a functionalization efficiency of 70% for AA into chitosan nanoparticles [24].
Figure 1. Representative phase-contrast images and size distribution histograms of (a) Fe$_3$O$_4$ NPs and (b) Fe$_3$O$_4$@CS-AA NPs, obtained by AFM.

3.2 Structural characterizations of Fe$_3$O$_4$ NPs and Fe$_3$O$_4$@CS-AA NPs

Figure 2A shows the XRD patterns of Fe$_3$O$_4$ NPs (uncoated NPs) and Fe$_3$O$_4$@CS-AA NPs. It can be observed the presence of six characteristic peaks attributed to magnetite (Fe$_3$O$_4$) (JCPDS 20-596), indicating that magnetite is the only state of iron oxidation in the nanomaterial [3,25]. The results demonstrated that after coating Fe$_3$O$_4$ NPs with CS and AA, the crystalline phase of Fe$_3$O$_4$ NPs remained unchanged [18]. The grain diameters of Fe$_3$O$_4$ NPs and Fe$_3$O$_4$@CS-AA NPs, obtained by the Debye-Scherrer equation, were 9.05 nm and 10.50 nm, respectively. Our results are in accordance with crystallite sizes from 10 to 11 for Fe$_3$O$_4$ coated with glutathione and PEG [3].
Figure 2. (a) XRD patterns of uncoated Fe$_3$O$_4$ NPs (red line) and Fe$_3$O$_4$@CS-AA NPs (black line). (b) FTIR spectra of Fe$_3$O$_4$ NPs (black line), Fe$_3$O$_4$@CS-AA NPs (red line), pure CS (blue line), and pure AA (green line), in the range of 4000 a 500 cm$^{-1}$. (c) Fe 2p high resolution spectrum for Fe$_3$O$_4$ NPs obtained by XPS measurement. (d) Exploratory spectrum of uncoated Fe$_3$O$_4$ NPs, reporting the constituents of the chemical structure of the magnetite represented by Fe 2p, O 1s and C 1s.

Figure 2B shows the FTIR spectra of Fe$_3$O$_4$@CS-AA NPs, in comparison with Fe$_3$O$_4$ NPs, pure CS and pure AA. Peaks attributed to Fe$_3$O$_4$ core can be observed at 578 cm$^{-1}$ in the Fe$_3$O$_4$@CS-AA NPs and at 622 cm$^{-1}$ in the uncoated Fe$_3$O$_4$ NPs. The presence of CS and AA on the surface of the Fe$_3$O$_4$ NPs is supported by peaks at 3456 cm$^{-1}$ (OH) and 1650 cm$^{-1}$ (C = O). The results showed the successful coating of Fe$_3$O$_4$ NPs with CS and AA. Unsoy et al. showed similar stretches to FTIR spectra for Fe$_3$O$_4$ NPs and Fe$_3$O$_4$@CS-AA NPs [26].

Figure 2C shows the exploratory spectrum of uncoated Fe$_3$O$_4$ NPs. Peaks associated with O 1s, Fe 2p and C 1s were observed. All the elements observed belong to the chemical structure of the Fe$_3$O$_4$ NPs, except for the C 1s peak, which is associated with the presence of gaseous species adsorbed on the surface of the nanoparticles, commonly observed in the XPS technique [27]. Figure 2D shows the high resolution spectrum of Fe2p for the Fe$_3$O$_4$ NPs, where it was possible to determine the Fe$^{3+}$ to Fe$^{2+}$ ratio present in the sample. This ratio can be used as an indication the quality of the synthesis, where values close to 2 indicate that there is only or mainly the magnetite phase in the sample and values higher than 2 indicate the possible oxidation of the magnetite in another material. Another factor that suggests that the phase of the samples correspond to the magnetite is the position of the Fe2p$_{1/2}$, satellite and Fe2p$_{3/2}$ peaks (indicated in Figure 2D), which should be positioned at 725, 719
and 711 eV, respectively. The XPS analysis corroborates the data shown by Cuenca et al., confirming the composition of Fe₃O₄ NPs obtained in the present work [27].

3.3 Magnetic characterization of Fe₃O₄ NPs and Fe₃O₄@CS-AA NPs

The superparamagnetism is an important feature for and efficiency target drug delivery system [5,28]. Figure 3 shows the magnetic hysteresis curve of (a) Fe₃O₄ NPs and (b) Fe₃O₄@CS-AA NPs at 300 K. The hysteresis loops for both, Fe₃O₄ NPs and Fe₃O₄@CS-AA NPs, confirmed the superparamagnetic behavior (Figure 3A and B). The observed saturation magnetization (MS) values were 77.84 emu⁻¹ for Fe₃O₄ NPs and 77.65 emu⁻¹ for Fe₃O₄@CS-AA NPs, both at 300 K (Figure 3A and B). It has been reported that the MS tends to decrease when Fe₃O₄ NPs are coated with polymers, since there is an exchange of electrons between the Fe atomic surface and polymer coating [6,7]. However, the observed results in this work showed that after coating the Fe₃O₄ NPs with CS and AA no significant decrease in MS was observed for the nanoparticles, indicating that the coating used did not significantly change the superparamagnetic behavior of Fe₃O₄ NPs. The magnetization results showed that Fe₃O₄ NPs and Fe₃O₄@CS-AA NPs present superparamagnetic behavior at room temperature, which is desirable for biomedical applications.

3.4 In vitro AA release from Fe₃O₄@CS-AA NPs

The in vitro vertical diffusion cell technique was used to evaluate the diffusion profile of AA from Fe₃O₄@CS-AA NPs, in comparison with free AA (Figure 4). The release profile of AA from Fe₃O₄@CS-AA showed a linear increase of AA concentration in the first 20 h, followed by the establishment of a steady-state of AA diffusion for at least 48 h. In contrast, the free AA showed a burst of AA diffusion in the first 5-6 h followed by a quickly decrease of AA concentration related to AA decomposition. Thus, the functionalization of nanoparticle surface with CS/AA have a suitable profile for drug release with a sustainable drug release up to 48 h. Similar profiles were observed for Ding et al. using Fe₃O₄ NPs with cyclodextrin [20]. The results obtained in this study for the Fe₃O₄@CS-AA NPs indicated that these NPs have potential in drug delivery therapies.
Figure 4. *In vitro* AA diffusion from Fe$_3$O$_4$@CS-AA NPs (black line) compared with free AA (red line) at 25° C for 48 h. The initial AA concentration was 30 mg mL$^{-1}$ for both groups. The results are presented as mean ± standard error of two independent experiments.

3.5 Cytotoxicity of Fe$_3$O$_4$ NPs, Fe$_3$O$_4$@CS NPs and Fe$_3$O$_4$@CS-AA

Figure 5 shows the percentage of cell viability of human breast epithelial cells (MCF-10A), a non-tumor cell line [29], after incubation with Fe$_3$O$_4$ NPs, Fe$_3$O$_4$@CS NPs and Fe$_3$O$_4$@CS-AA NPs, at different concentrations. An increase in cell viability was observed in a non-concentration dependent manner. The highest increase in cell viability for all nanoparticles was observed at 30, 100 and 500 μg mL$^{-1}$. It should be noted that the three groups of nanoparticles did not present significant cytotoxicity to the tested epithelial cells, indicating the biocompatibility and low toxicity of nanoparticles. These results indicate that Fe$_3$O$_4$ NPs, Fe$_3$O$_4$@CS NPs and Fe$_3$O$_4$@CS-AA NPs are suitable for biomedical applications in drug delivery system.
Figure 5. Percentage of viability of human breast epithelium (MCF-10A) incubated with Fe$_3$O$_4$ NPs, Fe$_3$O$_4$@CS NPs and Fe$_3$O$_4$@CS-AA NPs. Cell viability was estimated by a tetrazolium-based reduction assay (MTT). Results are presented as percentage of control (absence of NPs) as mean ± standard error of two independent experiments.

4. Conclusions

Fe$_3$O$_4$ NPs coated with CS were successfully synthesized. The results showed the formation of 50-60 nm nanoparticles with CS layers that contributes to avoid agglomeration of particles and oxidation of the metal core. The CS layer also provides an anchoring site for AA (drug model). The Fe$_3$O$_4$@CS-AA NPs showed sustainable release of AA for at least 48 h. Structural analysis confirmed the presence of Fe$_3$O$_4$ core of the nanoparticles with superparamagnetic behavior, before and after CS coating. The cytotoxicity assay of Fe$_3$O$_4$ NPs, Fe$_3$O$_4$@CS NPs and Fe$_3$O$_4$@CS-AA NPs did not demonstrate significant toxicity against human breast epithelial cells (MCF-10A). Taken all together, Fe$_3$O$_4$@CS-AA NPs have promising features for target drug delivery therapies.

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