Biocompatible superparamagnetic nanoparticles with ibuprofen as potential drug carriers

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Abstract

This article describes the synthesis, characterization, in vitro cytotoxic and genotoxicity evaluation of chitosan-iron oxide nanoparticles (Fe_3O_4 -CS) as vehicles for ibuprofen (IBU) molecule. Magnetite (Fe_3O_4) nanoparticles were synthesized by co-precipitation of iron salts and coated with chitosan, leading to the formation of Fe_3O_4 -CS hybrid nanoparticles, and then IBU was adsorbed on the surface of the modified nanoparticles. The physicochemical, morphological, and magnetic properties of the nanoparticles were determined by X-ray powder diffraction, Fourier transform infrared spectroscopy, scanning electron microscopy, transmission electron microscopy and SQUID magnetic measurements. The nanoparticles have shown stability in aqueous medium presenting an average hydrodynamic size of 36 ± 9 nm for Fe_3O_4 -CS, and 132 ± 1 nm for Fe_3O_4 -CS-IBU. The cytotoxicity of nanoparticles using the cell viability of the blood mononuclear cell fraction and the analysis of gene expression of the repair genes hMSH2, hMSH6 and NF- κ B were performed to evaluate their applicability as drug carriers. The results showed that Fe_3O_4 -CS nanoparticles are suitable candidates as magnetic vehicles for ibuprofen in biomedical applications.

Graphic abstract

Keywords Iron oxide · Nanoparticles · Biocompatibility · Chitosan · ibuprofen

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1 Introduction

There is a growing interest about new vehicles for drugs in biomedical applications that can reduce undesirable toxic side effects and improve the pharmaceutical efficacy [1–5]. Among various vehicles superparamagnetic iron oxide nanoparticles (SPIONs), magnetite (Fe_3O_4) have been widely employed due to their wide range of biomedical applications [6–11].

Since these nanoparticles present superparamagnetic behavior, they can be guided to target sites by applying an external magnetic field, which allows the control over their biodistribution [12, 13]. In addition, these nanoparticles are much less toxic than their correlative materials [14–16].

As SPIONs can act locally, adsorbed or incorporated drugs can be released in the required target [17, 18]. Particularly, surface coating of NPs with biocompatible molecules based on a *core-shell* system is an interesting approach to avoid aggregation in aqueous media [19]. There are several molecules suitable to functionalize SPIONs surfaces [17, 20–22] such as thiol groups from mercaptosuccinic acid (MSA), dimercaptosuccinic acid (DMSA) [10, 23, 24] glutathione (GSH), cysteine (Cys) [24–26], and silica [27, 28]. Vinluan et al. [22] has shown that the surface modification with Glutathione (GT), an endogenous antioxidant composed of glutamic acid, cysteine and glycine, increases the physiological stability and agglomeration resistance of the NPs to certain proteins. In addition, polymers, such as polyethylene glycol (PEG), [21, 29] also increase the physiological stability and can be used as coordination sites for biomolecules.

Furthermore, NPs covering with biocompatible biopolymers such as chitosan, a cationic biopolymer at lower pHs, has attracted particular attention since it presents low toxicity, mucoadhesive properties, a flexible structure, and hydroxyl and amino groups with strongly affinity with water [19, 30–32]. Chitosan (CS), has been extensively researched in recent years as a primary material in the formation of carriers for therapeutic proteins and as non-viral gene-carrying vehicles [33–35], being therefore used in several pharmaceutical applications [33].

Coating nanoparticles with CS promote not only an increase of the biocompatibility of these systems, but also represent a strategy to conjugate active molecules to the nanoparticle surface. Particularly, drug delivery processes are employed to carry important active molecules in a region-specific manner by attaching them to the large surface area of the magnetic nanoparticle, releasing the drug in the biological system directly at the local target [36, 37]. The CS groups on the nanoparticles

surface can interact with biomolecules and release them directly in the target. Zowalaty et al. [38] have developed streptomycin (Strep)–chitosan (CS) magnetic nanocomposites aiming antibacterial activity. They have reported that SPIONs with CS layer must prevent aggregations, increase the colloidal stability, and drugs can be incorporated in the system to using it as a vehicle for drug delivery.

Among the several drugs of pharmaceutical interest, ibuprofen (Fig. 1) is a non-steroidal anti-inflammatory compound able to reduce inflammation, pain and fever, being also used in the treatment of rheumatoid arthritis, osteoarthritis, tendonitis, among other diseases [39, 40]. This drug irreversibly inhibits the enzyme cyclooxygenase 1 (COX1), which converts arachidonic acid to prostaglandin, the lipid responsible for inflammatory processes. This action protects the stomach and intestine by the action of two other enzymes, generating thromboxane, responsible by platelet aggregation, and prostacyclin. Ibuprofen can also lead to bleeding, renal dysfunction, ulcers, and undesirable effects in the nervous system, among others. A controlled release of this drug helps diminish the side effects of drugs in the body.

In this study, iron oxide (Fe_3O_4) magnetic nanoparticles were synthesized and coated with chitosan leading to biocompatible nanoparticles. Ibuprofen molecule was incorporated on the NPs surface leading to a new vehicle Fe_3O_4 -CS-IBU. The physical-chemical, morphology and magnetic properties of the compounds were characterized by different techniques. Tests were carried out to evaluate the cytotoxicity of the compounds, such as cell viability and analysis of the genotoxicity.

2 Methods

2.1 Synthesis of Fe_3O_4 nanoparticles

Nanoparticles were synthesized by co-precipitation method [21, 41]. In brief, 4 mL of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (1 mol L^{-1}) and 1 mL of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (0.5 mol L^{-1}) prepared in 1 mol L^{-1} of HCl were mixed and stirred at room temperature, while a volume of 50 mL of 0.7 mol L^{-1} of NH_4OH was slowly added. The precipitate was magnetically decanted and

Fig. 1 Ibuprofen molecular structure

washed five times with ethanol, thus resulting in the formation of Fe_3O_4 nanoparticles.

2.2 Preparation of Fe_3O_4 nanoparticles coated with chitosan- Fe_3O_4 -CS

Chitosan (CS) dissolved in acetic acid 1% was added to a suspension of Fe_3O_4 nanoparticles in deionized water (mass ratio 1:5), and the mixture was stirred for 1 h, leading to the formation of Fe_3O_4 -CS nanoparticles. The nanoparticles were isolated by centrifugation and washed five times with ethanol [42].

2.3 Adsorption of ibuprofen on the surface of Fe_3O_4 -CS nanoparticles: Fe_3O_4 -CS-IBU

It was added 0.4 g of ibuprofen to 4 mL of a chitosan in acetic acid solution and, after that, the mixture was stirred for 2 h, followed by the addition of an aqueous (1%) dispersion of Fe_3O_4 nanoparticles. The mixture was stirred for one hour. The final dispersion was centrifuged and washed several times with deionized water, leading to Fe_3O_4 -CS nanoparticles covered with ibuprofen molecule (Fe_3O_4 -CS-IBU).

2.4 Characterization of the magnetic nanoparticles

2.4.1 X ray diffraction (XRD)

X-ray powder diffraction data were collected on a STADI-P diffractometer (Stoe®, Darmstadt, Germany), in transmission geometry (the samples were deposited between two acetate-cellulose foils), operating at 50 kV and 40 mA, using $\text{MoK}\alpha_1$ radiation ($\lambda = 0.7093 \text{ \AA}$) selected by a curved $\text{Ge}(111)$ crystal. The X-ray photons were detected by a Mythen 1 K detector (Dectris®, Baden, Switzerland) in the range from 5° to 100° (2θ) with a step size of 0.015° (2θ) and integration time of 120 s per 3.15° . Assuming a Gaussian superposition, the average crystallite size of each nanoparticle was calculated from the full width at half maximum of reflections, by Scherrer's equation [43].

2.4.2 Fourier transform infrared (FTIR) spectroscopy

Nanoparticles were triturated with pure KBr powder, and a hydraulic press was used to make the pellets while extracting the air with a mechanical pump. The measurements were performed on a Shimadzu Prestige-21 spectrometer. The FTIR spectra were recorded from 400 to 4000 cm^{-1} at a resolution of 2 cm^{-1} .

2.4.3 Magnetic measurements

Magnetic properties were performed using a super-conductive quantum interference device (SQUID) magnetometer, model MPMS XL7, of the Quantum project, at a constant temperature of 300 K. All measurements were made on dried powder, slightly pressed and packed into Lucite cylindrical holders.

2.4.4 Zeta potential (ELS) and dynamic light scattering (DLS)

The DLS measurements were performed using an ALV/CGS-3 compact goniometer system consisting of a 22 mW HeNe linearly polarized laser operating at a wavelength of $\lambda = 633 \text{ nm}$, an ALV 7004 digital correlator and a pair of avalanche photodiodes operating in the pseudo-cross-correlation mode. The samples were loaded in 10-mm-diameter glass cells, dispersed in deionized water using a concentration of 1 mg mL^{-1} , diluted in 20 mL of water and the temperature was maintained constant ($25 \pm 1^\circ \text{C}$). Autocorrelation functions were obtained in the angular region of 90° and adjusted by using the cumulant method [44]. ELS measurements were used to determine the average zeta potential (ζ) of the nanoparticles which were collected using a Zetasizer Nano-ZS ZEN3600 instrument (Malvern Instruments, UK). This instrument measures the electrophoretic mobility (U_E) and converts the value to ζ -potential (mV). The sample preparations were the same at DLS.

2.4.5 Scanning electron microscopy (SEM) and energy dispersive X-ray fluorescence spectrometry (EDS)

Nanoparticles morphology was performed by scanning electron microscopy (SEM) (JEOL model JSM-6010LA) at a voltage of 30 kV and in high vacuum, with an energy dispersive X-ray fluorescence spectrometry (EDS) module. The EDS technique was employed for the semi-quantitative and qualitative microanalysis of the sample elements and chemical mapping of nanoparticles surface.

2.4.6 Transmission electron microscopy (TEM)

Images of the nanoparticles were acquired using a Philips CM200 transmission electron microscope (TEM) with an energy dispersive spectrometer, operating at 160 kV. The samples were dispersed in water, and then a drop of the supernatant dispersion was deposited on an amorphous carbon film supported by a copper grid.

The size distribution and the average diameter were calculated from 250 nanoparticles.

2.4.7 Quantification of ibuprofen adsorbed on the surface of Fe₃O₄-CS-IBU

Ibuprofen on the surface of nanoparticles was quantified by UV–Vis spectrophotometry, based on the absorbance at 230 nm [36]. Briefly, 0.1 mg of Fe₃O₄-CS-IBU nanoparticles were dispersed in 1700 µL of 1 × TBE buffer (pH 8) containing 2 mmol L^{−1} of ethylenediaminetetraacetic (EDTA) acid. The dispersion was placed into a quartz cuvette, and the absorption band at 230 nm was measured using a UV–Vis spectrophotometer (Agilent 8453). The experiments were conducted in triplicate with the estimated standard deviation evaluated.

2.5 Biological experiments

2.5.1 Cell viability

The samples used were collected following good laboratory practices from healthy donor. For the analysis it was used peripheral blood collected in the K3EDTA vacuum tube. The cell viability/mortality was assessed by the MTT assay (a colorimetric assay to assess cellular metabolic activity) using SIGMA® 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. The compounds were analyzed at 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.62, 5.0, 2.5, 1.0, 0.5, 0.1 µg L^{−1} concentrations.

To separate the mononuclear cells fraction to the other blood cells the Ficoll Hypaque technique, which is a density gradient centrifugation, was performed. In addition, the Neubauer chamber to count and to obtain the final concentration of 1 × 10⁵ cells per 1 mL was used.

The cells were added in Dulbecco's Modified Eagle's Medium (DMEM) culture medium (200 µL) and added 20 µL of each compound to the wells of the plate and then were incubated for 24 h in an oven at 37 °C in a 5% CO₂ atmosphere. In a second step, 100 µL of MTT solution in PBS at a concentration of 0.5 mg mL^{−1} was added per well of the plate. The cells were incubated for 2 h at 37 °C and after that 200 µL of pure dimethyl sulfoxide (DMSO) were added to all slots. The plate was shaken for 3 min until the formazan crystals disappeared. Furthermore, the absorbance at 540 nm was measured on the Adaltis Labotech equipment. The results were submitted to 1-way ANOVA using GraphPad PRISM software and $P < 0.05$ was the significance value. After reading, 100 µL of the Trizol reagent was added for RNA extraction.

2.5.2 Genotoxicity

The RNA extraction was obtained from cells used in cell viability/mortality analysis. The concentration of the compounds used for gene expression analyses was obtained from a pool of the concentrations used in the previous essay, (approximately 307.19 µg mL^{−1}). The expression of the repair genes hMSH2 (homologue MutS 2), hMSH6 (homologue MutS 6) and NF-κB (nuclear factor kappa B) was analyzed, which acts as a set of processes that make the cell to identify and correct the damage of molecules of the DNA that encode your genome. It was determined by real-time quantitative polymerase chain reaction in duplicate using SYBR Green Master Mix (Invitrogen®). The internal control was the ribosomal protein gene L13a (RPL13A). The reaction was performed with 20 ng cDNA in a 20 µL reaction containing 1 × SYBR Green (Invitrogen), 0.25 µM from each primer. The sequences of primers were used, for hMSH2, *Forward*-CCTTGTAACCTTCATTTGATCC and *Reverse*-ATCCAACTGTGCACTGGA, amplification of 157 for hMSH6 gene. *Forward*-GAACATTCATCCGCGAGAAA and *Reverse*-TGAGGGCTCATCACA AACTG amplification of 250. NFκB gene, *Forward*-CTCTGT CATTCTGTGCTTCCA and *Reverse*-CATCCCATGGTGGACTAC CT, amplification of 286. For gene RPL13a, *Forward*-TTGAGG ACCTCTGTGTATTTGTCAA and *Reverse*-CCTGGAGGAGAA GAGGAAAAGGA, amplification of 126. The amplification reaction was performed in an Applied Biosystems 7500 Polymerase Chain Reaction - Real Time systems thermocycler, with an initial incubation 95 °C for 2 min, followed by 40 cycles for 15 s at 95 °C, 60 s at 60 °C, and 15 s at 72 °C. The relative gene expression was determined by the ΔCt method. The results were submitted to one-way analysis of variance (ANOVA) using GraphPad PRISM software and $P < 0.05$ was the significance value and for normality evaluation was the Shapiro–Wilk statistical analysis.

3 Results and discussion

3.1 Synthesis of Fe₃O₄-CS nanoparticles

Several methods to obtain Fe₃O₄ are described in the literature [17, 45]. The purpose of this investigation has been focused on the preparation of magnetite nanoparticles by chemical co-precipitation method [46]. Magnetite nanoparticles can be successfully prepared with particle sizes in the range from 10 to 40 nm by controlling the reaction condition. In addition, a higher molar ratio of ferric ion to ferrous ion (2–1) was used in the co-precipitation method because the magnetite particle size can be reduced by increasing the ion ratio due to the formation of ferric hydroxide [47]. It is a low-cost route, which allows good

Fig. 2 Powder X-ray diffraction patterns for Fe_3O_4 -CS (blue line) (a) and Fe_3O_4 -CS-IBU (red line) (b) nanoparticles. The diffractograms are displaced for better visualization

control over the size, distribution, and morphology of SPIONs dispersed in water at room temperature. The co-precipitation reaction is represented by Eq. (1):

3.2 X-ray diffraction (XRD)

The XRD patterns of the Fe_3O_4 -CS and Fe_3O_4 -CS-IBU samples are shown in Fig. 2, indicating the absence of iron hydroxides, and confirming the magnetic core of the nanoparticles. However, the spinel crystal structure of magnetite is very similar to that of maghemite ($\gamma\text{-Fe}_2\text{O}_3$) and SPIONs are often a mixture of magnetite and maghemite [44]. Nonetheless it was observed that the diffraction profile is consistent to the standard pattern to a spinel-like crystalline structure of the magnetite (Fe_3O_4) (JCPDS#: 110614).

The peaks identified below at approximately 10° can be associated to the crystalline structure of ibuprofen [48]. Therefore, no significant differences in the reflection peaks between the two nanoparticles were observed. Crystallite sizes of both nanoparticles were obtained from Scherrer's equation [43, 49] by taking into account the (311) Bragg's reflection. The magnetic core diameters for Fe_3O_4 -CS and Fe_3O_4 -CS-IBU were calculated to be 13 nm and 19 nm, respectively. Therefore, XRD analysis confirms the existence of an iron oxide core.

3.3 Fourier transform infrared (FTIR) spectroscopy

Figure 3a–d shows the FTIR spectra of pristine chitosan (CS), ibuprofen (IBU) and functionalized Fe_3O_4 -CS and Fe_3O_4 -CS-IBU nanoparticles. Figure 3a shows the main

Fig. 3 FTIR spectra of chitosan (CS) (a), ibuprofen (IBU) (b); Fe_3O_4 -CS (c) and Fe_3O_4 -CS-IBU (d)

bands assigned, in dash lines, to chitosan biopolymer at 3440 cm^{-1} ($\nu\text{O-H}$ and $\nu\text{N-H}$ from the amine group) (1), 1630 cm^{-1} ($\delta\text{N-H}$) (2), 1407 cm^{-1} ($\nu\text{C-N}$) (3) and in the region of 1024 cm^{-1} ($\nu\text{C-O-C}$) (4) [50–53]. In addition, Fig. 3b presents the bands attributed to ibuprofen molecules at 2957 cm^{-1} (5), at 1720 cm^{-1} (6), at 1508 cm^{-1} (7), at 1230 cm^{-1} (8) and at 780 cm^{-1} (9) assigned to $\nu\text{C-H}$, $\nu\text{C=O}$, $\nu\text{C=C}$, $\nu\text{C..C}$ and δCH_2 , respectively. Figure 3c shows the Fe_3O_4 -CS functionalized nanoparticles in which the presence of the biopolymer on the surface is supported by components labelled as (1), (2), (3) and (4) in the same region. Moreover, the iron oxide core can be observed at 590 cm^{-1} ($\nu\text{Fe-O}$) (10). The slight changes in the components (2) and (3) when compared with the pristine chitosan reveals the interaction between biopolymer and SPIONs. Furthermore, concerning nanoparticles with ibuprofen anchored, Fe_3O_4 -CS-IBU, the components (5), (6) and (9) indicate the presence of ibuprofen on the nanoparticle surface, as shown in Fig. 3d. Although the capping was evidenced, these bands practically remains unchanged indicating that Fe_3O_4 -CS are unaffected with the ibuprofen presence. These results evidence the successful formation of Fe_3O_4 -CS-IBU nanoparticles, in accordance with a previous study [54].

3.4 Magnetic measurements

Due to the importance of superparamagnetism for the delivery of magnetic targeting nanocarriers, magnetic characterizations were carried out. Hysteresis isothermal (300 K) curves are shown in the Fig. 4 for Fe_3O_4 -CS and Fe_3O_4 -CS-IBU. In both samples, superparamagnetic

Fig. 4 Magnetization curves at room temperature for: Fe_3O_4 -CS (blue line) (a) and Fe_3O_4 -CS-IBU (red line) (b)

behavior is evidenced. The saturation magnetization (M_s) values commonly found in the literature for magnetic nanoparticles are usually smaller than those for the corresponding bulk phases, which are $\sim 90 \text{ emu g}^{-1}$ [16, 28]. The values for the present work are around 60 emu g^{-1} to Fe_3O_4 -CS nanoparticles. It was possible to note a lower magnetization value for the nanoparticle containing IBU molecule. There are two possible causes for the decrease in the magnetization value upon the presence of an extra IBU coating. The first one is, the presence of another diamagnetic layer besides the chitosan; and the second one is the contributions from intrinsic particles, shape and stress anisotropies due to different ligands along with interparticle interactions (dipolar), which can provide a difference in the energy barrier. Nanoparticles are strongly attracted by a magnetic field, and when this field is removed, their magnetization is reduced close to zero, without residual magnetization [54–56].

Fig. 5 Scanning electron microscopy image for: Fe_3O_4 -CS (magnification of 1590) (a) and Fe_3O_4 -CS-IBU (magnification of 1603) (b)

3.5 Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

Figures 5a, b show the SEM images of Fe_3O_4 -CS and Fe_3O_4 -CS-IBU, respectively. It can be observed that the nanoparticles have approximately a spherical morphology. The composition of the nanoparticles was evaluated by EDS. Figure 6a shows SEM images for nanoparticles containing IBU, in which the light green dots corresponds to iron (Fe, from magnetite core), dark green corresponds to oxygen (O, from magnetite, chitosan and ibuprofen) and red dots correspond to carbon (C, from chitosan and ibuprofen) atoms.

The weighted percentages of Fe, O and C (Fig. 6b) were found to be 28.1%, 38.7% and 33.2%, respectively. As expected, Fe atoms are homogeneously dispersed on the nanoparticles. A homogeneous distribution of dots in the islands of C and O atoms can be observed in the mapped area.

Figures 7a, b shows TEM photographs of Fe_3O_4 and Fe_3O_4 -CS-IBU respectively, corroborating with that previously observed in the SEM images. The spherical-like morphology is an important feature that can facilitate the carriage of NPs in bloodstream. NPs agglomeration is also observed in solid state, but this characteristic may change in colloidal dispersions.

As a result, ibuprofen was successfully incorporated on iron nanoparticles - chitosan surfaces, showing a potential use of these supramolecular system as drug delivery systems for biomedical applications.

3.6 Zeta potential (ELS) and dynamic light scattering (DLS)

As the hydrodynamic size of nanoparticles may affect their properties, we determined the particles size by using the DLS technique, which usually shows particle diameters significantly higher than those obtained at solid state determined by XRD analysis [57]. This effect is assigned to the presence of extra hydrate layers in aqueous medium

Fig. 6 Scanning electron microscopy images in which light green dots corresponds to Fe, dark green corresponds to O and red dots correspond to C (**a**). Elemental analysis showing EDS peaks of iron (Fe), oxygen (O), and carbon (C) atoms (**b**)

Fig. 7 TEM photographs of Fe_3O_4 (**a**) and Fe_3O_4 -CS-IBU (**b**)

Table 1 Structural features of the chitosan-iron oxide nanoparticles

NPs	R_H (nm)	μ_2/Γ^2	ζ (mV)
Fe_3O_4 -CS	36 ± 9	0.39 ± 0.04	-15 ± 1.8
Fe_3O_4 -CS-IBU	132 ± 1	0.19 ± 0.01	-13 ± 0.1

[21]. In addition, some agglomeration of the nanoparticles in aqueous solutions is expected due to the high surface free energy.

DLS and zeta potential (ζ) measurements were performed in the physiological pH value (7.4) and are shown in Table 1. The degree of NPs hydration showed a

significant increase after the conjugation with IBU ($RH > 3\times$), and the hydrodynamic size modification corroborates the FTIR data related to the IBU synthesis and adsorption process on the NPs. On the other hand, the presence of IBU conferred a reduction in the index of polydispersity, which is associated to the greater stability of the colloidal system. Since the hydrodynamic radius provides the nanoparticle size considering interactions with water molecules, when IBU is attached on the nanoparticle surface, its size increases and consequently the hydrodynamic radius [58, 59].

3.7 Quantification of ibuprofen adsorbed on the surface of SPIONs

To quantify the ibuprofen molecule on the nanoparticle surface, a calibration curve was performed with the

Fig. 8 Calibration curve with IBU solutions in TBE buffer (pH 8) for quantification of adsorbed ibuprofen on nanoparticles surface

Fig. 9 Graphs cell viability (%) of mononuclear fraction of compounds Fe_3O_4 , CS, IBU, Fe_3O_4 -CS and Fe_3O_4 -CS-IBU at concentrations 2000, 250, 5, 1, 0.1 $\mu g mL^{-1}$ (* in the graph represent significant differences with $p < 0.05$). CTRL is the control

solutions of ibuprofen ($50\text{--}250 \mu g mL^{-1}$), as explained in Methods Section. The curve presented a value of ϵ equal to $3.64 L mmol^{-1} cm^{-1}$ (Fig. 8). The number of mols of IBU adsorbed on the surface of NPs was calculated from Beer-Lambert law and it was $1.92 mmol$ of ibuprofen per grams of nanoparticles.

A pH value of 8 for the Fe_3O_4 -CS-IBU solution is highly consistent for the intravenous loading and release of this drug. Besides the blood pH being slightly basic (approximately 7.3 or 7.4), the hydrophobic and ionic interactions between the cationic polysaccharide and ibuprofen are broken under these conditions, supporting the release process.

3.8 In vitro assays

The cell viability studies were performed using the mononuclear blood cell fraction added with Fe_3O_4 , Fe_3O_4 -CS and Fe_3O_4 -CS-IBU compounds. As shown in Fig. 9, it is possible to observe that all compounds analyzed at concentrations up to $250 \mu g mL^{-1}$ showed viability greater than 60%. At $2000 \mu g mL^{-1}$, Fe_3O_4 -CS and Fe_3O_4 -CS-IBU showed values above 60%, while Fe_3O_4 nanoparticles and CS and IBU molecules showed viability below 50%. Thus, these results show that this new system containing CS and IBU on Fe_3O_4 surface presents potential bioapplications, such as drug administration in high concentrations, when compared to the others analyzed.

Ibuprofen is a non-steroidal anti-inflammatory agent (not derived from hormones) that inhibits the production of prostaglandins (substances that stimulate inflammation) which generates anti-inflammatory (reduces inflammation), analgesic (reduction, even suppression, pain) and antipyretic (reduction, even suppression, fever) activity. Ibuprofen is absorbed in the gastrointestinal tract and the peak concentration plasma occurs around 1–2 h after ingestion. Ibuprofen is largely bound to plasma

proteins and has a half-life of approximately 2 h. It is rapidly excreted in the urine mainly as metabolite and its conjugates. Approximately 1% is excreted in the urine as unchanged Ibuprofen and about 14% as conjugated Ibuprofen. Ibuprofen is rapidly metabolized and eliminated in the urine; The excretion is practically complete 24 h after the last dose. The main source of metabolism is the liver, where Ibuprofen is converted by hydroxylation and carboxylation of the isobutyl group into inactive metabolites. There is a combination of Ibuprofen and arginine on the market to increase absorption (reported above) and decrease side effects also reported. Thus, the new chemical system proposed in this article (Fe_3O_4 -CS-IBU) can, in the future, be compared to this combination.

The compounds with the highest toxicity were Fe_3O_4 and CS, that had EC_{50} values below $500 \mu\text{g mL}^{-1}$ (Table 2) and therefore were not suitable for higher concentrations. However, after combination with the other compounds, toxicity decreased, indicating that this bond decreases toxicity and are better options than the separate use of Fe_3O_4 and CS.

This new system is an attractive option for preclinical trials with other cell lines to analyze new possible therapeutic actions, as the isolated effects of CS and IBU compounds are already known and widely researched [60–63].

The genes hMSH2 and hMSH6 are a part of the DNA mismatch repair (MMR), which were characterized in 1993 by using PCR fragments, with the first human MMR gene

being hMSH2. The human MMR system is then known to be composed of four heterodimers: MutS α , MutS β , MutL α and MutL β [64, 65].

Particularly the MutS α heterodimer is formed by hMSH2 and hMSH6 proteins, and MutS β heterodimer consists of hMSH2 and hMSH3 proteins. Also, MutL α heterodimer is formed by hMLH1 and hPMS2 proteins and MutL β heterodimer is formed by hMLH1 and hMLH3 proteins [66, 67]. The high and constant expression by hMSH2 and hMSH6 genes shows a much higher risk of developing some types of neoplasms compared to the general population that did not express them, as is known as repair genes, a process that needs to be constantly working to quickly resolve defect in DNA structure [68].

There was no expression of hMSH2 gene for CS, indicating low genotoxicity when compared to the other compounds and the control. In the evaluation of the hMSH6 gene, expression occurred in all compounds because this gene is part of the incompatible base repair system and studies describe that their levels are regulated according to the cell cycle phase, when increasing mRNA levels increase during the G1 phase, this does not necessarily mean an increase in the amount of protein specific for repair, therefore, the expression of repair genes should be evaluated together [69, 70].

The hMSH2 protein is known to stabilize hMSH6 due to existing as heterodimers, so elevations in hMSH6 levels that is not accompanied by its hMSH2 pair result in the degradation of excess free hMSH6 [69–71], as seen in compound CS (Fig. 10a, b).

It was also evaluated, the NF- κ B heterodimer gene, discovered in 1986, a protein complex that performs functions as a transcription factor [72]. The NF- κ B is a transcription factor, which is described as a master regulator for inflammatory response and is related to tumor development [68, 69]. Including neoplasms for example as breast cancer, stomach, leukemia, and lymphoma cancer, they

Table 2 EC_{50} values for all compounds

Compounds	EC_{50} ($\mu\text{g.mL}^{-1}$)
Fe_3O_4	414.41
CS	319.81
IBU	699.71
Fe_3O_4 -CS	708.29
Fe_3O_4 -CS-IBU	530.21

Fig. 10 Graphs of gene expression of hMSH2 (a), hMSH6 (b) and NF- κ B (c) (Δ Ct) compounds Fe_3O_4 , CS, IBU, Fe_3O_4 -CS, Fe_3O_4 -CS-IBU and control (** in graph represents absence of gene expression)

have been described with NF- κ B as a key regulator of their development and progression [73–75].

Some researches show positive feedback between NF- κ B stimulation and some signaling that provides tumor progression, such as up-regulation of cyclooxygenase (COX)-2, nitric oxide synthase (NOS), inflammatory cytokines such as IL-6, IL-8 and TNF and chemokines such as CCL2 and CXCL8 [74, 76, 77].

Normally their elevated expression is observed [78, 79] compared to the other genes analyzed and their control (Fig. 10c). This superiority is caused by the various stimuli that activate it, such as the numerous genes and phenomena that NF- κ B regulates and is found in almost all animal cell types and is involved in the cellular response to stimuli such as stress, cytokines, free radical, ultraviolet radiation and viruses [80, 81].

The fact that cells with CS do not present expression indicates that this molecule cannot be considered dangerous to health, immune system and, therefore, inflammatory and repairing. Moreover, when it is not stimulated, NF- κ B factor is found in the cytoplasm associated with an I κ B inhibitor protein. This complex prevents translocation of NF- κ B to the nucleus, so phosphorylation and I κ B degradation are required for translocation to occur [80].

However, it has been observed that some studies are associating tumor risk factors with the expression of the hMSH2 and hMSH6 repair genes with NF- κ B transcription factor in several types of cancer [82]. Thus, our results show that the compounds in general present low cytotoxicity and genotoxicity, especially for Fe₃O₄-CS and Fe₃O₄-CS-IBU, demonstrating potential application in drug administration.

4 Conclusions

This work described the synthesis of magnetite nanoparticles by the co-precipitation method and the coating of their surfaces with chitosan polymer (CS) (Fe₃O₄-CS) as carrier vehicles of ibuprofen molecule, (Fe₃O₄-CS-IBU). XRD patterns and FTIR spectra confirmed the composition of capped nanoparticles and showed that the successful coating of Fe₃O₄ with these biomolecules and no phase changes were observed. Superparamagnetic behavior at room temperature also has been evidenced. TEM, SEM and EDS analysis demonstrated the spherical morphology and the composition of the nanoparticles, while DLS revealed a good behavior in physiological dispersion of Fe₃O₄-CS and Fe₃O₄-CS-IBU. An amount of 3.64 L mmol⁻¹ cm⁻¹ was found to be the quantity of ibuprofen on Fe₃O₄-CS-IBU surfaces. These compounds showed low toxicity and genotoxicity for mononuclear cells analyzed at low concentrations and the evaluation of hMSH2, hMSH6 and NF- κ B gene

expression showed no damage to the genetic material repair system and also not affect the transcription factor. Finally, the results showed that the new compound Fe₃O₄-CS-IBU, is a good candidate for biomedical drug delivery applications.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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