Potential Use of DMSA-Containing Iron Oxide Nanoparticles as Magnetic Vehicles against the COVID-19 Disease

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1. Experimental Section

1.1. Preparation of SPIONs by the coprecipitation method

Fe₃O₄ nanoparticles were synthesized using a co-precipitation method, as previously reported [1,2]. Briefly, 4.0 mL of FeCl₃·6H₂O and 1.0 mL of FeCl₂·4H₂O (molar ratio 2:1), prepared in 1.0 mol·L⁻¹ HCl, were mixed, and stirred, while a volume of 50 mL of NH₄OH (0.7 mol·L⁻¹) was added as precipitator. At this stage, the black suspension formed was decanted magnetically. After removing the SPIONs from the aqueous medium, 7 mL of oleic acid was dropped onto the SPIONs surface and then stirred for 20 min. The SPIONs were decanted, and the new precipitate was washed several times with ethanol, leading to nanoparticles covered with oleic acid [3].

1.2. Surface functionalization with dimercaptosuccinic acid (DMSA)

In an aqueous medium, oleic acid can stabilize SPIONs only through the formation of a bilayer stabilizing their surface. This bilayer is destroyed when the SPIONs are taken in an organic solvent [4]. Therefore, oleic acid-coated Fe₃O₄ was suspended in toluene while DMSA was dissolved in dimethyl sulfoxide (DMSO). The two mixtures were blended and vigorously stirred for 14 h producing a black powder that was decanted magnetically. This procedure led to ligand exchange and, hence, to the formation of water-stable DMSA-containing nanoparticles (DMSA-Fe₃O₄) in mass ratio Fe₃O₄:ligand 1:5; 1:10 and 1:30.

1.3. Determination of the thiol groups on the surface of SPIONs

The number of thiol groups (–SH) on the surface of the nanomaterials was measured by the 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) reaction, based on the absorbance at 412 nm (ε = 14,150 mol·L⁻¹·cm⁻¹) of the 2-nitro-5-thiobenzoate anion (TNB⁻) generated in the reaction of –SH groups with DTNB [5]. DMSA-SPIONs were added to DTNB (0.01 mol·L⁻¹) in phosphate-buffered saline (PBS) buffer (pH 7.4) containing 1 mmol·L⁻¹ of ethylenediaminetetraacetic acid. After 5 min of incubation, the suspensions were filtered using 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 a UV-Vis Spectrophotometer (Agilent, model 8553, Palo Alto, CA, USA). The experiments were carried out in triplicates.

1.4. Characterization of the magnetic nanoparticles

1.4.1. 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 λ = 633 nm, an ALV 7004 digital correlator, and a pair of avalanche photodiodes operating in the pseudo-cross-correlation mode. The samples were loaded in glass cells, dispersed in deionized water using a concentration of 1 mg·mL⁻¹, diluted in 20 mL of water at room temperature. Autocorrelation functions were obtained in the angular region of 90° and adjusted by using the cumulant method [6]. ELS measurements were used to determine the average zeta potential of amounts which were collected using a Zetasizer Nano-ZS ZEN9600 instrument (Malvern Instruments, UK).

1.4.2. Fourier transform infrared (FT-IR) spectroscopy

Dry DMSA-Fe₃O₄ nanoparticles were mixed with pure potassium bromide (KBr) and the measurements were executed using the Agilent Cary 630 spectrophotometer. The spectra were obtained from 400 to 4000 cm⁻¹ with a resolution of 4 cm⁻¹. A pure KBr pellet was used as a background.

1.4.3. 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 and using MoKa1 radiation (λ = 0.7093 Å) selected by a curved 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 0.01° (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 [6].

1.4.4. Transmission electron microscopy (TEM)

Images of the DMSA-Fe₃O₄ 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.

1.4.5 Magnetic measurements
Magnetization measurements of DMSA-Fe$_3$O$_4$ were performed using a superconducting quantum interference device (SQUID) magnetometer, model MPMS XL7, from Quantum Design at 300 K, at Multiuser Experimental Center (CEM) of the Federal University of ABC (UFABC). The measurements were performed on dried powders, which were slightly pressed and conditioned in lucite cylindrical holders.

1.5. COVID-19 contaminated cells preparation and DMSA-SPIONs (1:10) up-take

To compare and analyze the cytotoxicity of the studied compounds, whole blood samples donated from healthy patients and patients with positive results were used for the Anti-SARS-CoV-2 IgA Elisa test (EURO IMMUN), conducted by the laboratory of the University Center Health of ABC (Committee of the ABC Medical College 2028/12229-0).

This test provides a semi-quantitative in vitro assay for human IgA class immunoglobulin antibodies against SARS-CoV-2 in serum or plasma with EDTA, heparin, or citrate to support the diagnosis of SARS-CoV-2 infection and is a complement to direct detection of the pathogen. It has a sensitivity of 100% and a specificity of 92.5% ten days after the onset of symptoms.

Samples from patients were selected because they are described as markers of acute infection in respiratory diseases, with the appearance of IgA and IgM antibodies occurring approximately five days after the onset of symptoms, while IgG antibodies can be detected in 14 days after first symptoms.

1.5.1. In vitro assays

According to the International Standard Organization, ISO 10993, the cytotoxicity test should be the first assay to evaluate the toxicity of the samples tested and when the results are satisfactory, the samples can be conducted to in vivo phase [7,8].

1.5.2. Mononuclear fraction cytotoxicity

To compare and analyze cytotoxicity of the studied nanoparticles, donated whole blood from healthy patients and from patients with positive results for the Anti-SARS-CoV-2 IgA ELISA test were used. The whole test is described in Fig. S1.

To separate the mononuclear fraction, the Ficoll Hypaque® technique was performed. Two groups with 10.0 mL of blood in EDTA were used to analyze cells with positive and negative COVID-19. The blood was added into Falcon® tube with 10.0 mL of 0.9% NaCl solution. In another Falcon® tube with 3.0 mL of Ficoll-Paque®, 7.0 mL of the blood sample was slowly added resulting in two phases. The experiments were carried out in quadruplicates.

In the next step, the tubes were centrifuged at 1500 rpm for 30 minutes, leading in a sample separated into 4 phases, from the top to the bottom: plasma, mononuclear fraction, Ficoll-Paque®, and red cells and granulocytes. The mononuclear fraction was removed from all tubes, re-suspended in 10.0 mL of 0.9% NaCl solution, and centrifuged for 10 minutes at the same rotation previously used. After centrifugation, the supernatant was discarded, and the procedure was carried out 2 other times to obtain a sample with the only lymphocyte and remove the remnants of Ficoll-Paque® since it has high toxicity that could lead to cell lysis. The counting of collected lymphocytes was done using a Neubauer Chamber. The sample was diluted to obtain a final concentration of 1x10^5 cells.mL$^{-1}$.

The nanoparticles were dispersed in RPMI-1640 culture medium from Roswell Park Memorial Institute to reach the following concentrations 0.1, 5.0, 50, 500, and 2000 μg.mL$^{-1}$. In a 96-well flat-bottomed microplate, in triplicate, 200.0 μL of the cell...
suspension was added with 20.0 µL of culture medium and 20.0 µL of nanoparticles dispersion. The plate was incubated for 24 hours. 

After this period, the cytotoxicity assay was performed by MTT colorimetric assay. To carry on this assay, 500.0 µL of dimethylthiazolyl-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich; 1 mg. mL\(^{-1}\)) made in PBS was added in each well of the plate. The plate was incubated for 4 hours leading to the formation of formazan purple crystals that were dissolved with dimethylsulfoxide (DMSO). Absorbance measurements at 540 nm were carried on ELISA and the cell viability was calculated as a ratio between sample and control values. 

1.5.3 Statistical Analysis

Statistical analysis was performed by GraphPad Prism Software version 7.0 (GraphPad Inc., CA, USA) using normality evaluation as the Shapiro–Wilk, one-way ANOVA, and Tukey’s test. The threshold for significance was P = 0.05 and P values < 0.05 (*). 

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