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WATER SOLUBLE MAGNETITE NANOPARTICLES FOR ANTIMICROBIAL DRUGS DELIVERY

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Abstract

Water-soluble magnetite has been prepared through precipitation approach. These nanoparticles coated with sulfanilic acid could be dispersed in hydrated aqueous systems. The product was characterized with X-ray powder diffraction (XRD), Dynamic Light Scattering (DLS) and the in vitro efficacy as antibiotic delivery vehicles as well as their influence on the eukariotic cells. The XRD pattern confirm the product to be Fe_3O_4. The nanoparticles with average size 10.45 nanometers are not cytotoxic and do not influence the eukariotic HeLa cell cycle, representing potential tools for the delivery of drugs in a safe manner. Water soluble magnetite improves the activity of currently used antibiotics, representing potential as a nanocarrier for these antimicrobial substances, to achieve extracellular and intracellular targets.

Keywords *Water soluble magnetite, magnetic nanoparticle, drug targeting, drug delivery, flowcitometry*

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Introduction

Fe_3O_4 nanoparticles have attracted great attention for many important biomedical applications such as magnetic separation [1], drug delivery [2], cancer hyperthermia [3], inhibition of biofilm formation on prostethic devices [4], stabilization of volatile organic compounds [5], insulin delivery, cell imaging, and antidiabetic research [6], due to their non-toxicity property and high chemical stability [7]. Iron oxide nanoparticles in combination of external magneticfield allow delivering particles to the desired target area and fixing them at the local site while the pharmaceutical drug is released and acts locally [8,9]. In magnetic drug targeting systems, the general approach is to employ an external magnet positioned near a target site located at some depth below the skin to attract and retain the magnetic drug carrier particles[10]. *Pseudomonas aeruginosa*, a gram-negative aerobic bacterium with minimal nutritional requirements, is common in most environments [11]. It is naturally resistant to many antibiotics and has a remarkable capacity for acquiring new resistance mechanisms under selective pressures from antibiotics, creating increased therapeutic problems [12]. It rarely causes infection in healthy humans but may do so following disruption of physical barriers and in patients with certain underlying illnesses [13]. *Pseudomonas aeruginosa* is a leading cause of nosocomial infections and is responsible for 10% of all hospital acquired infections [14]. *Staphylococcus aureus* is among the most important nosocomial pathogens because of both

the diversity and the severity of the infections it causes, including superficial, deep skin, and soft -tissue infections, endocarditis, and bacteremia, as well as a variety of toxin-mediated diseases such as gastroenteritis, staphylococcal scalded-skin syndrome, and toxic shock syndrome [15]. These infections can be further complicated by treatment failures, due to the increasing occurrence of multi-drug resistant *S. aureus* strains [16]. With all these in mind, the purpose of this work was the fabrication, characterization and *in vitro* evaluation of novel drug loader system based water soluble magnetite and different antibiotics against *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 strains.

Experiment Details

Synthesis of water soluble magnetic nanoparticles. Magnetite is usually prepared by wet chemical precipitation [17] from aqueous iron salt solutions by means of alkaline media, like HO^- or NH_3. In the present paper, core/shell nanospheres were prepared by a modified precipitation method [18,19]. One gram of sulfanilic acid was solubilized in a known volume of distilled-deionized water, corresponding to a 1.00% (w/w) solution, under stirring at room temperature. Then, 4 mL of a basic aqueous solution consisting of 28% NH_3 were added to sulfanilic acid solution. After these, 100 mL aqueous solution of 0.6 g $FeCl_3$ and 1.2 g$FeSO_4X7H_2O$ were dropped under permanent stirring up to pH = 8, leading to the formation of a black precipitate. The product was repeatedly washed with methanol, separated with a strong NdFeB permanent magnet and subsequently dried in oven at 60 °C until reaching a constant weight.

XRD. X-ray diffraction analysis was performed using a Shimadzu XRD 6000 diffractometer at room temperature. In all the cases, Cu Kα radiation from a Cu X-ray tube (run at 15 mA and 30 kV) was used. The samples were scanned in the Bragg angle 2θ range of 10-80.

DTA-TG. The differential thermal analysis (DTA) coupled with thermo gravimetric analysis (TGA) was performed with a Shimadzu DTG-TA-50H, at a scan rate of 10^oC/min, in air.

Antimicrobial activity assay. An adapted diffusion method was used in order to assess the influence of the water soluble nanovehicle to the antimicrobial actitivity of VA (vancomycin), DA (clindamycin), AZM (azithromycin), OX (oxacyllin), SXT (trimethoprim/ sulfamethoxazole), RA (rifampicin), OFX (ofloxacin), TE (tetracycline), P (penicillin), CIP (ciprofloxacin), GM (gentamicin), TZP (piperacillin/ tazobactam), FEP (cefepime), ATM (aztreonam), CAZ (ceftazidim) and PRL (piperacillin) against *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 strains. The tested antibiotics have been chosen according to CLSI recommandations. Standardized antibiotic discs have been placed on the Mueller Hinton agar medium distributed in Petri dishes previously seeded with a bacterial inoculum with a density corresponding to the 0.5 McFarland standard. Five µL of the stock solutions of the water soluble nanovehicle were spoted over the antibiotic disks. The plates were incubated 24h at 37°C, and the inhibition zones diameters for each antibiotic, after the addition of the tested nanomaterial suspensions were quantified and compared with the growth inhibition zones obtained for the respective antibiotics.

Biocompatibility assay.

Fluorescence microscopy. $5x10^5$ HeLa cells were seeded in each well of 24 well plate. After 24 hour, the cells were treated with water soluble nanoparticles in the final concentration 100 µg/ml, 500µg/ml. The effect of water soluble nanoparticles was evaluated after 24 hour by adding 100 µl PI (0.1mg/ml) and 100 µl fluorescein diacetate (FdA). Fluorescence was quantified using Observer.D1 Carl Zeiss microscope.

Apoptosis detection. Flow cytometry analysis was performed to discriminate between intact and apoptotic cells using FITC-labeled annexin-V, and propidium iodide (Annexin V-FITC Apoptosis Detection Kit I, BD Bioscience Pharmingen, USA), according to manufacturer's protocol. Briefly, total cells ($1×10^6$ cells) were resuspended in 100 µl of binding buffer and stained with 5µl Annexin V-FITC and 5µl propidium iodide for 10 minutes in dark chamber. For each sample, at least 10,000 events were acquired using a

Beckman Coulter flow cytometer and were analyzed using FlowJo software.

Results and Discussions

The crystalline structure was characterized by XRD. As shown in Figure 1, the magnetite (Fe_3O_4) was identified in the sample as the only one crystalline phase (the main peaks of magnetite are centered at 2θ = 30.31, 35.71, 43.31, 57.61 and 62.81). Based on the intensity of the characteristic peaks of magnetite no preferential directions of crystallization were identified [20].

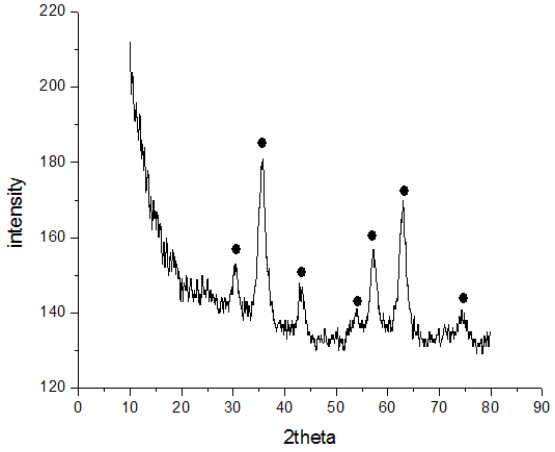

***Figure 1**: XRD pattern of water soluble magnetite*

The synthesized water soluble magnetite was characterized function of its thermogravimetrical behavior up to 1000°C. Three main processes are discernible. The first process (endothermic process) is attributed to water evaporation (25-170°C) and leads to a weight loss of 2.21%. The second two processes are attributed to the sulfanilic acid degradation. The first process (exothermal) is accompanied by a weight loss of 1.89% while the second exothermal process is accompanied by a lower weight loss (~1.3%) but it must mention that over this range of temperature magnetite is also oxidized at hematite and consequently these mass losses are apparent, and could not be used for quantifying purposes. Assuming that magnetite transformation to hematite as well as sulfanilic acid degradation is complete (up to 1000°C), based on the Eq. 1 it can estimate that magnetite content is ~ 89.09%. The sulfanilic acid content is estimated based on the content of water and magnetite being ~8.7%.

$$\frac{100}{\underset{2\cdot 232}{2\ Fe_3O_4}} + 1/2\ O_2 \rightarrow \frac{103.45}{\underset{3\cdot 160}{3\ Fe_2O_3}} \qquad \textit{Eq. 1}$$

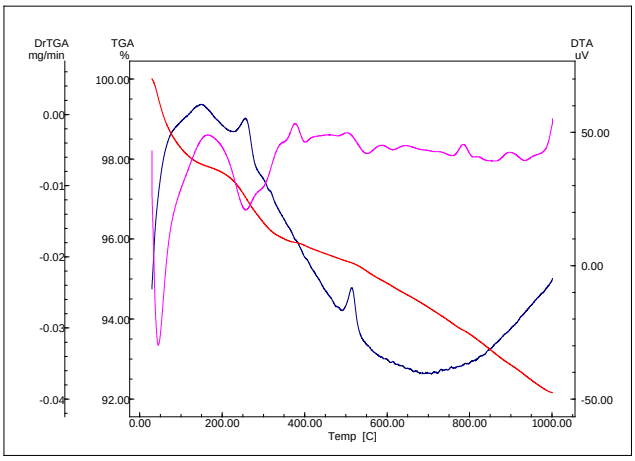

***Figure 2**: Thermal analysis of water soluble magnetite*

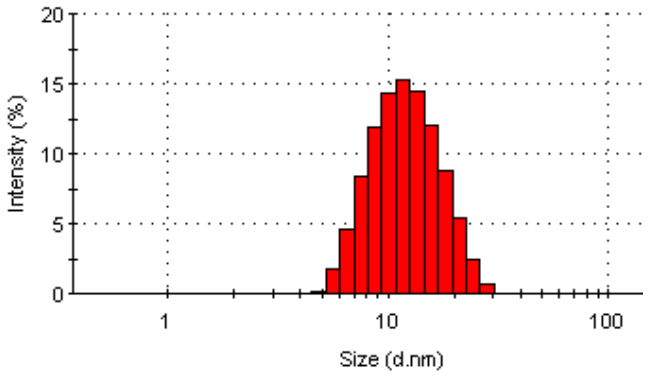

***Figure 3**: Size distribution histogram*

The average size of the individual water soluble magnetite nanoparticles is about 11.43 nm, according to DLS results (Figure 3).

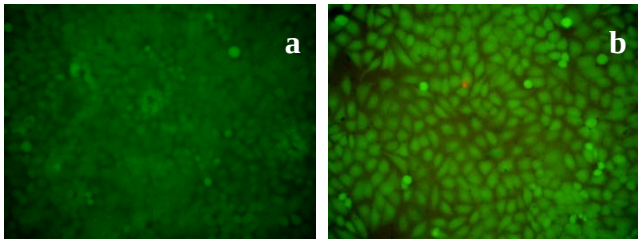

***Figure 4**: The effects of different concentrations of water soluble magnetite on HeLa cells: 100µg/ml (a) and 500µg/ml (b) (fluorescent microscopy, 100x).*

Regarding the toxicity, we observed only a very low percentage of apoptotic cells induced by 500µg/ml water soluble magnetite nanoparticles, quantified by microscopy (red cells) and flow cytometry.

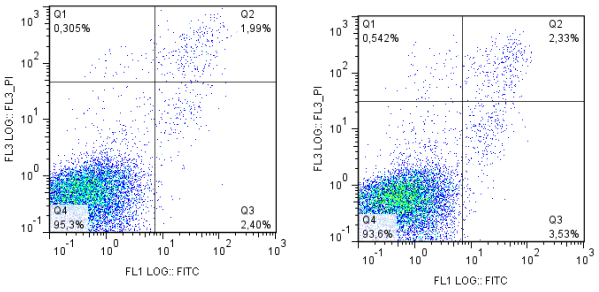

Figure 5: *Histrograms showing the measurement of HeLa apoptosis/necrosis rates after 24 hours treatment with 100µg/ml (a) and 500µg/ml (b) of water soluble magnetite nanoparticles. Apoptotic cells (FITC+/PI-, fluorescent green), necrotic or late apoptotic cells (FITC+/PI+, fluorescent red and green), and viable cells (FITC-/PI-, non fluorescent).*

Concerning the ability of the tested nanosystems to carry and deliver antibiotics in active forms, in case of *P. aeruginosa*, the incorporation of the anti-pesudomonal antibiotics in the nanoparticles led in all tested cases, excepting the piperacillin +tazobactam, to an increase of the bacterial growth inhibition diameters (Figure 6), demonstrating that the tested nanosystem could represent an useful carrier for these antibiotics. In case of *S. aureus* strain, the potentiation of the antimicrobial activity of different antibiotics was limited to four of the tested spectrum, i.e. penicillin, oxacillin, aztreonam and doxycycline (Figure 7). However, it is to be mentioned that in the other antibiotics case, the incorporation into the tested nanosystem did not affect the antibiotic efficiency, which remained the same as in the case of antibiotic controls.

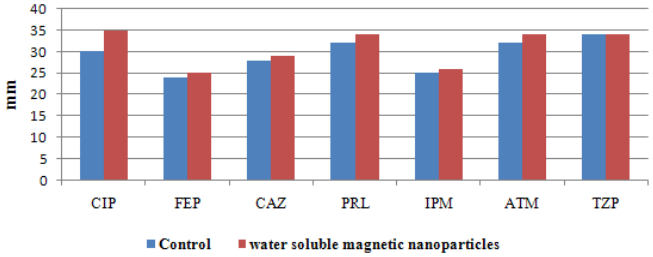

Figure 6*: The growth inhibition zone diameters (mm) obtained for the tested antibiotics in the presence of magnetite nanoparticles on the P. aeruginosa ATCC 27853 strain*

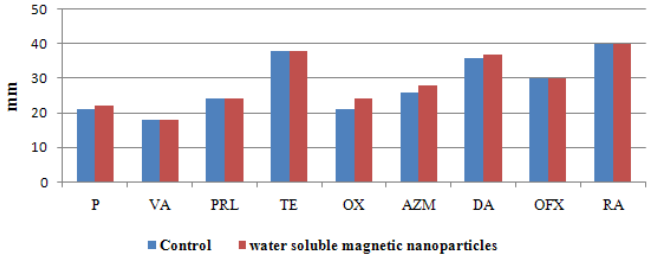

Figure 7*: The growth inhibition zone diameters (mm) obtained for the tested antibiotics in the presence of magnetite nanoparticles on the S. aureus ATCC 25923 strain*

Conclusions

Our results recommend the water soluble magnetite nanoparticles for applications in the biomedical field. They improve the activity of currently used antibiotics belonging to penicillins, macrolides, aminoglycosides, rifampicines and quinolones classes, representing thus potential nanocarriers for these antimicrobial substances. The obtained nanoparticles with average size 11.43 nm and identified by XRD as magnetite are not cytotoxic and do not influence the eukariotic HeLa cell cycle, representing thus an alternative for the development of safe drug delivery systems.

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