HIGHLY BIOCOMPATIBLE MAGNETITE NANOPARTICLES FUNCTIONALIZED WITH CHITOSAN FOR IMPROVING THE EFFICIENCY OF ANTIBIOTICS

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In this study we report on the synthesis, characterization and antimicrobial activity of a newly engineered nanobiosystem containing magnetite nanoparticles functionalized with sulfanilic acid, chitosan and antibiotics. The obtained nanoparticles proved an enhanced antimicrobial effect, significantly potentiating the activity of the antibiotics neomycin and erythromycin. The results also demonstrated that sulfanilic acid may enhance the antimicrobial effect of magnetite nanoparticles, while chitosan confers a good biocompatibility to the system. Obtained data support the idea that this bioactive nanosystem may be efficiently utilized in the treatment of severe infections, caused by resistant pathogens that require high, toxic amounts of antibiotics for eradication.

Keywords: magnetite nanoparticles, microbial pathogens, improved drug efficiency, antibiotics, chitosan

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1. Introduction

Nanotechnology has provided extensive ways of developing biomedical applications and recently has been utilized in the design of alternative antimicrobial strategies. Magnetite nanoparticles are the most investigated vectors utilized for improved drug delivery and controlled release of both natural and synthetic antimicrobials. Even though iron oxide nanoparticles have a low citotoxicity and good biocompatibility with the human body, it is recommended to utilize polymeric coatings for developing improved nanosystems with better delivery characteristics and improved biocompatibility. Chitosan (CS) is one of the most utilized polymers for the coating of nanoparticles intended for biomedical applications, especially in drug delivery and antimicrobial therapy. Recent studies revealed that nanoparticles coated with CS may switch the zeta - potential from negative to positive (which is able to promote cellular adhesion and retention of the delivery system at the target site), without necessary modifying the particle size distribution. Nanoparticles coated with CS also allow a significant modulation of *in vitro* drug release, providing a sustained drug delivery in cultured cells [1]. Chitosan nanoparticles have proved an increased antimicrobial effect *in vitro*, against *Streptococcus mutans* at neutral pH. Low molecular weight chitosan revealed an increased anti-biofilm effect, as compared with high molecular weight chitosan nanoparticles [2]. Zowalaty and coworkers revealed that streptomycin-loaded chitosan-coated magnetic nanocomposites possess antibacterial and anti-tuberculosis activities and have a more pronounced effect against Gram-negative than Gram-positive bacteria. Moreover, chitosan coated magnetite functionalized nanoparticles may be utilized for the successful fabrication of antimicrobial coatings. Our research group recently demonstrated that medical surfaces obtained by advanced laser techniques prepared with magnetite nanoparticles functionalized with *Melissa officinalis* extract and covered with poly lactic acid: chitosan coatings are able to significantly inhibit the biofilm formation of *Staphylococcus aureus* strains [3].

Nanoparticles are considered efficient tools for potentiating the antimicrobial activity of drugs and may be utilized for the treatment of severe infections caused by resistant pathogens. The fact that nanoparticles may reduce the necessary amount for eradicating resistant bacteria have been recently speculated and some studies reported the ability of nanoparticles to reduce the dose of the active drug, thus decreasing the side effects of antibiotics utilized in high amounts [4,5].

Sulfanilic acid is widely utilized as small molecular catalysts in the asymmetric synthesis of organic compounds and nanoparticles, being an efficient dispersing agent for magnetite nanoparticles [6]. Sulfanilic acid has been recently utilized for the development of functionalized silica-coated magnetite

nanoparticles being used as a reusable heterogeneous acid catalyst using a facile process [7].

Magnetite nanoparticles functionalized with chitosan, sulfanilic acid and antibiotics have not been reported to our knowledge, but the available literature regarding the properties of the nanoparticles, sulfanilic acid and chitosan independently or in different nanostructured combinations recommend this system as a successful nanostructured shuttle utilized for improved drug delivery.

In this context, the aim of this study was the synthesis, characterization and the assessment of biocompatibility and antimicrobial efficiency of a novel drug delivery system consisting of magnetite nanoparticles functionalized with chitosan, sulfanilic acid and antibiotics (neomycin and erythromycin).

2. Materials and Methods

2.1. Materials

Ferrous sulfate 7-hydrate ($FeSO_4 \bullet 7H_2O$), ferric chloride ($FeCl_3$), ammonia (NH_3, 25%), antibiotics (erythromycin, neomycin), chitosan (CS), sulfanilic acid (SA) were purchased from Sigma-Aldrich. All chemicals were of analytical purity and used with no further purification.

2.2. Preparation of nanoparticles

Magnetic nanostructures were prepared according to our previously published paper with a slight modification [8]. Sulfanilic acid was used in order to reduce the dimension of particles and to improve the antimicrobial activity of the prepared materials. 500 mL solution that contain deionized water, 1 g of chitosan, 5 mL acetic acid (1N), 1 g of $FeCl_3$ and 1.6 g of $FeSO_4$ x $7H_2O$ were dropped under permanent stirring into 300 mL aqueous solution that contain 10 mL NH_3 (25%) and antibiotics (erythromycin, neomycin). Obtained black precipitates were washed 3 times with deionized water (using a100kgf Nd-Fe-B external magnetic field) in order to remove the excess of antibiotics and other raw materials. After this step, the black precipitates were cross-linked with glutaraldehyde (1%).

2.3. Characterization

XRD

X-ray diffraction analysis was performed on a Shimadzu XRD 6000 diffractometer at room temperature. In all the cases, Cu Kα radiation from a Cu X-ray tube (run at 15mA and 30 kV) was used. The samples were scanned in the Bragg-Brentano geometry with 2θ angle range of 20-80 degree.

SEM

SEM analysis was performed on a FEI electron microscope, using secondary electron beams with energies of 30 keV, on samples covered with a thin gold layer.

TGA

The thermogravimetric (TG) analysis of the samples was followed with a Netzsch TG 449C STA Jupiter instrument (Netzsch, Selb, Germany). Samples were screened with 200 mesh prior to analysis, placed in an alumina crucible, and heated at 10 K min−1 from room temperature to 800 °C, under the flow of 20 mL min^{-1} of dried synthetic air (80% N2 and 20% O2).

FT-IR

IR spectra were recorded on a Nicolet iN10 MX FT-IR Microscope with MCT liquid nitrogen cooled detector in the measurement range 4000–700 cm-1. Spectral collection was made in reflection mode at 4 cm^{-1} resolution. For each spectrum, 32 scans were co-added and converted to absorbance using OmincPicta software (Thermo Scientific). Approximately 250 spectra were analyzed for each sample.

Cell viability

For cell viability assay, the endothelial cells (EAhy926 cell line, ATCC) were seeded in 96-well plates at a density of 5 x 10^3 cells/well in DMEM medium, supplemented with 10% fetal bovine serum. The cells were incubated with the nanosystems, represented by magnetite nanoparticles functionalized with antibiotics for 24 h; controls were represented by endothelial cells grown in the same culture conditions in the absence of nanoparticles. Fluorescent microscopy was assessed using a RED CMTPX fluorophore (Life Technologies, Invitrogen, USA), a long-term living cell tracker. The RED CMTPX dye was added in the culture medium at a final concentration of 5 μM, incubated for 30 minutes to allow the dye to penetrate the cells. The cells were washed with PBS (phosphate buffered saline) and visualized by fluorescence microscopy. The photomicrographs were taken with a digital camera using the Axio-Vision 4.6 (Carl Zeiss, Germany) software.

Antimicrobial assay

For establishing the MIC (minimum inhibitory concentration) values of the obtained functionalized magnetite nanoparticles we utilized a microdilution method performed in nutritive broth. The sterile broth was added in sterile 96 well plates and binary dilutions of each tested compound were performed in a final volume of 150 μL. After realizing the binary dilutions, 15 μL of microbial suspension adjusted to an optical density of 0.5 McFarland ($1.5x10^8$ CFU/mL) were added in each well. The MIC values were established by naked eye analysis and spectrophotometric measurement (Abs600nm). Each experiment was performed in triplicate and repeated on at least three separate occasions.

3. Results

The crystalline nature and phase analysis for prepared samples were confirmed by XRD analysis. The results are shown in Fig. 1. As it can be seen, the diffraction peaks of the samples match the typical diffraction pattern of magnetite (JCPDS no. 86-1354) [9]. The diffraction peaks are presented at 2θ = 30.1 °, 35.4 °, 43.1 °, 53.2 °, 56.9 °, and 62.5° and correspond to (2 2 0), (3 1 1), (4 0 0), (4 2 2), (5 1 1) and (4 4 0). In the case of Fe_3O_4/SA, a high purity sample was obtained, therefore no other peaks can be observed in the XRD pattern.

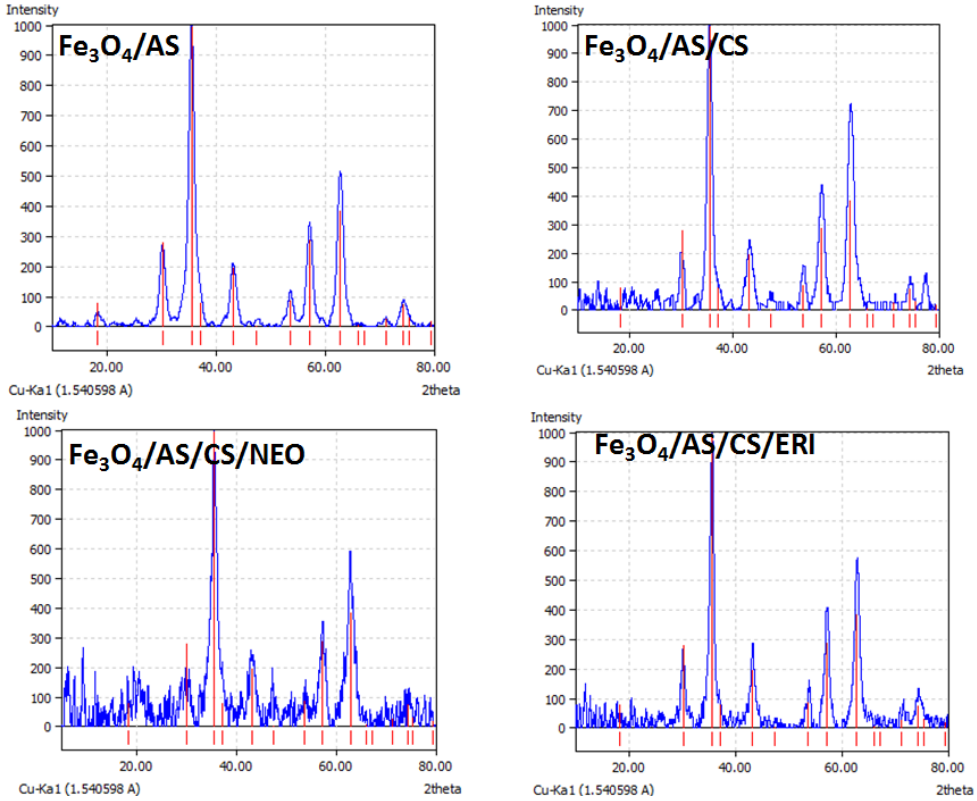

Fig. 1. XRD pattern of prepared samples

As shown in Fig. 2, magnetite nanoparticles functionalized with sulfanilic acid (Fe_3O_4/SA) showed a spherical shape with diameters lower than 8 nm with a high tendency to aggregate. In the case of Fe_3O_4/AS/CS and Fe_3O_4/AS/CS/ATBs, the tendency of aggregate is not observable; these samples presenting a better dispersion of nanoparticles in the polymer matrix. These data are suggesting that the presence of chitosan reduce the interaction between magnetite nanoparticles and correlate with previous findings [8].

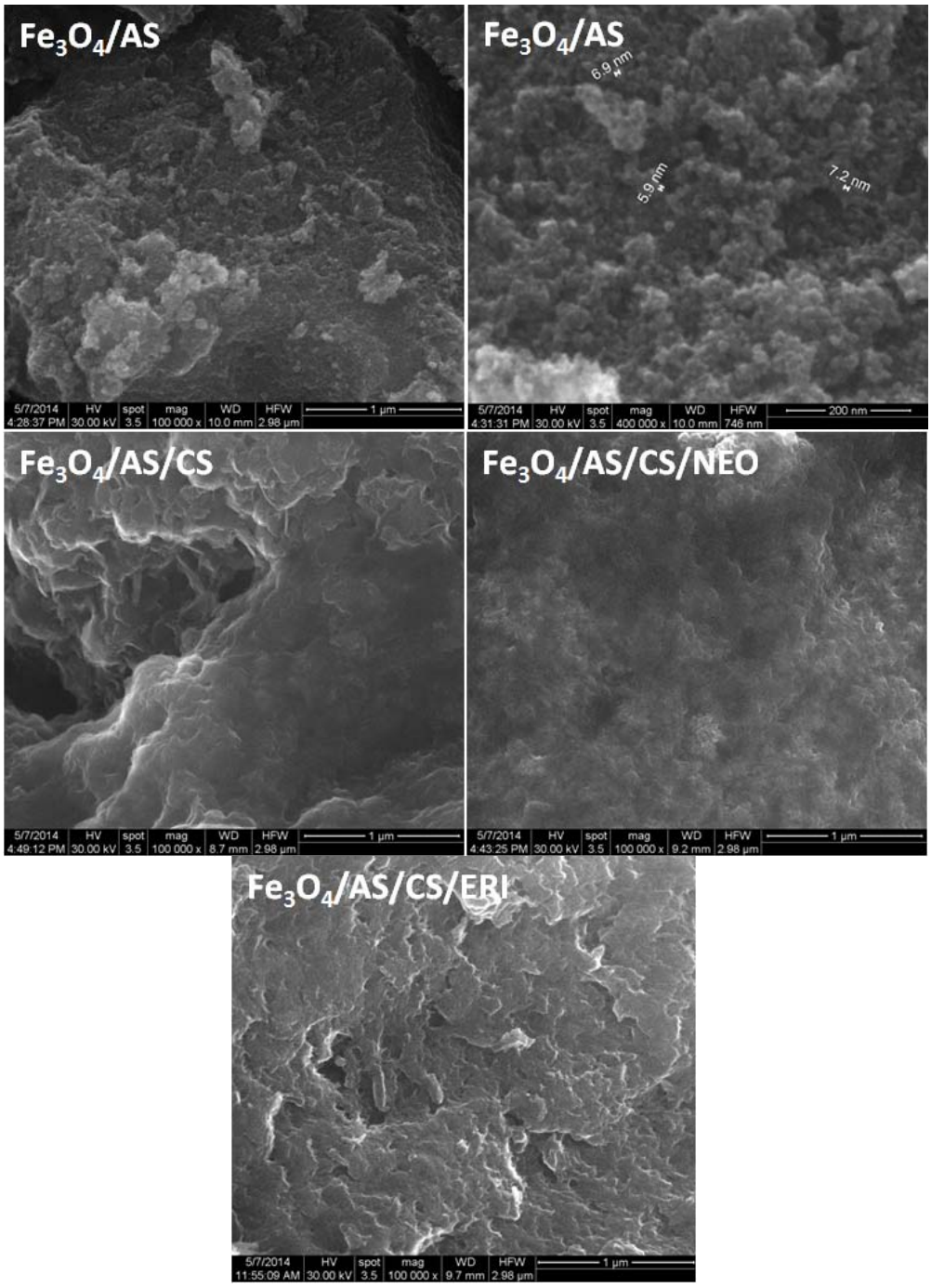

Fig. 2. SEM images of the prepared samples

The TGA analysis was used to estimate the amount of antibiotics entrapped in the polymer matrix during synthesis. The TGA curves of the uncoated Fe_3O_4 and coated samples with sulfanilic acid, chitosan and antibiotics are shown in the Fig. 3. The amount of antibiotics was estimated as a difference between the weight loss of Fe_3O_4/AS/CS and Fe_3O_4/AS/CS/ATBs. In this context the amount of ATBs was 1.76% in the case of erythromycin and 1.5% for neomycin.

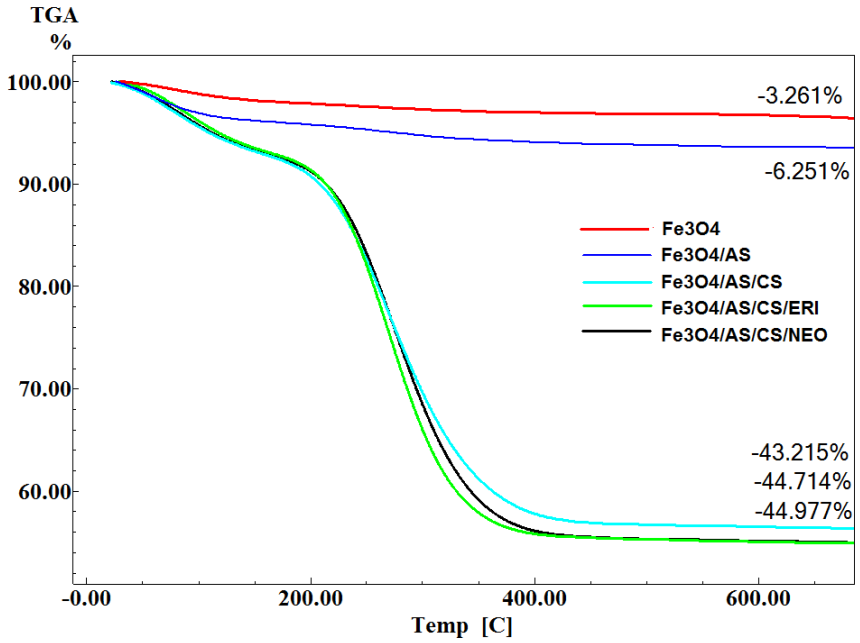

Fig. 3. TGA analysis of the samples

Infrared analysis is shown in the Fig. 4. According to Fig. 4 it can be concluded that the functionalization of magnetite nanoparticles was successfully achieved. IR spectra of Fe_3O_4/SA present absorption bands characteristic to sulfanilic acid as it follows: 3333 cm^{-1} assigned to OH, 2914 cm^{-1} assigned to CH and 1402 cm^{-1} is the characteristic peak of the benzene ring [6]. In the case of Fe_3O_4/AS/CS and Fe_3O_4/AS/CS/ATBs, absorption bands characteristic to chitosan can be identified: ~1020 cm^{-1} (stretching vibrations of CO single bonds of COH, COC and CH_2OH groups in the pyranose ring); ~1645 cm^{-1} (amide carbonyl groups from incomplete deacetylation of chitin); ~2914 cm^{-1} (stretching vibrations of CH single bonds in CH_2OH groups); ~3330 cm^{-1} (stretching vibrations of associated OH and NH_2 groups) [10]. The antibiotics were not identified in the IR spectra due to the low amount that was under the detection limit of the spectrometer.

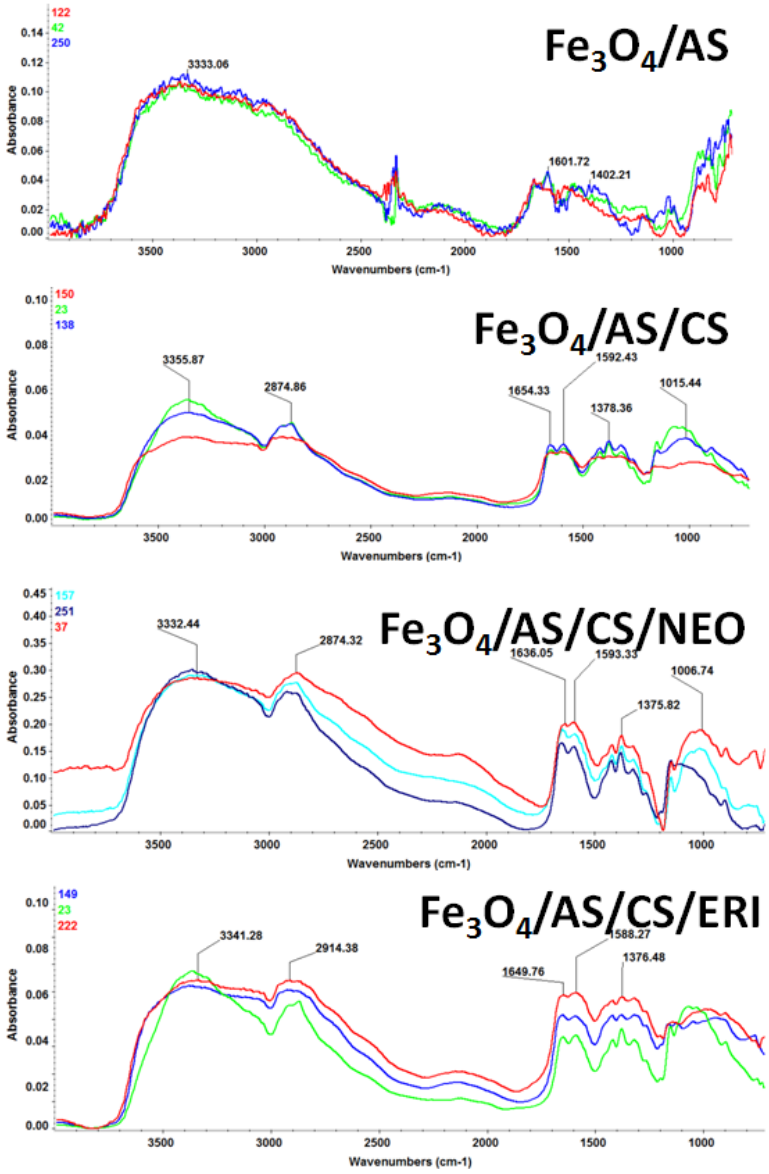

Fig. 4. IR spectra of prepared samples

The results obtained after the biological assessment showed that functionalized magnetite nanoparticles have different biological activities, being influenced by the content of chitosan and antibiotics. Regarding the cytotoxicity of the tested drug delivery nanostructured system, our results demonstrated that all

tested nanostructured systems containing functionalized magnetite nanoparticles have a good *in vitro* biocompatibility. Fluorescence microscopy analysis revealed that the growth and development of cultured human endothelial cells is supported by all obtained nanostructured materials. The microscopic aspect of the analyzed cells was normal, they manifesting all the characteristics of viable cells grown in control conditions (Fig. 5).

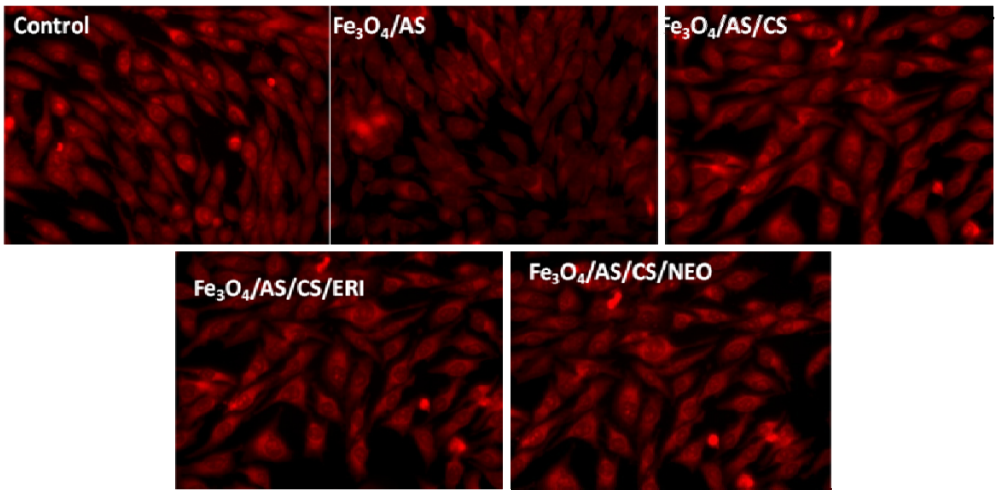

Fig. 5. Fluorescence microscopy images revealing the aspect of endothelial cultured cells grown in the presence of functionalized magnetite nanoparticles

The microbiology assay revealed that chitosan/antibiotics functionalized magnetite nanoparticles have an enhanced antimicrobial activity. The obtained nanosystems also potentate the activity of wide utilized antibiotics, such as neomycin and erythromycin up to 25 fold. The minimum inhibitory concentrations are strictly influenced by the functionalization of magnetite nanoparticles with sulfanilic acid, chitosan and antibiotics. In the case of *Escherichia coli* ATCC 8739 strain, the results demonstrated that the MIC values obtained for the magnetite nanoparticles functionalized with sulfanilic acid, chitosan and neomycin (Fe_3O_4/SA/CS/NEO) had the lowest value (0.039 µg/mL), while the MIC value obtained for Fe_3O_4/SA/CS was of 0,625 µg/mL, the MIC of Fe_3O_4/AS was 0.156 µg/mL and the MIC value obtained for the plain Neomycin solution was of 1 µg/mL (Fig. 6). Even though is not clearly documented, our results demonstrated that magnetite functionalized with sulfanilic acid are not cytotoxic *in vitro* in the case of human cultured cells.

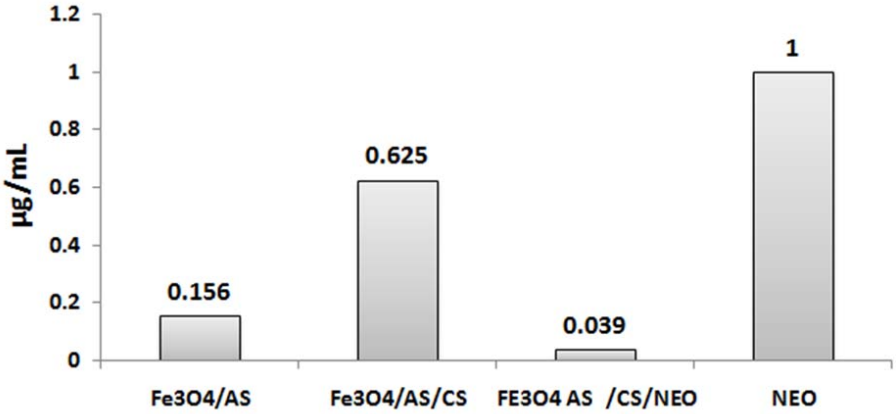

Fig. 6. Graphical representation of the MIC values obtained after the treatment of *E.coli* ATCC 8739 cultures with different concentration of the obtained magnetite nanoparticles functionalized with sulfanilic acid (AS), chitosan (CS), neomycin (NEO).

On the other hand, in the case of *Staphylococcus aureus* ATCC 25923, the results demonstrated that the MIC of the nanosystem functionalized with chitosan, sulfanilic acid and erythromycin has the lowest value (0.039 µg/mL). The MIC obtained for the *S. aureus* cultures treated with Fe_3O_4 coated with sulfanilic acid is 0.078 µg/mL; the value of MIC for Fe_3O_4 functionalized with sulfanilic acid and chitosan is 0.039 µg/mL, while the MIC of the samples treated with plain solution of erythromycin is 1 µg/mL (Fig. 7).

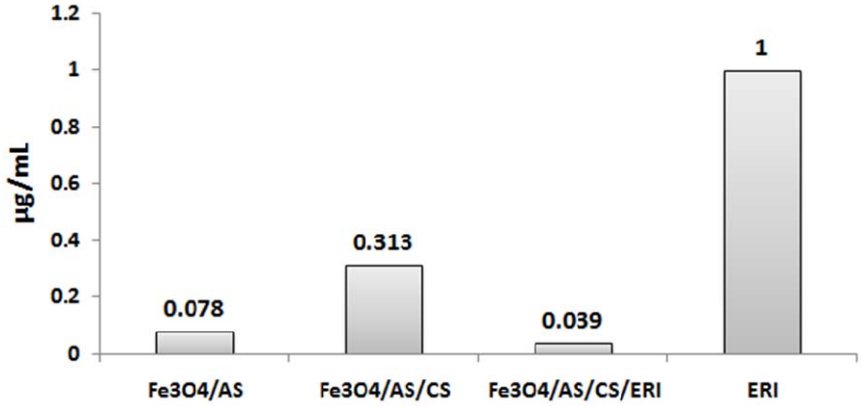

Fig. 7. Graphical representation of the MIC values obtained after the treatment of *S. aureus* ATCC 25923 cultures with different concentration of the obtained magnetite nanoparticles functionalized with sulfanilic acid (AS), chitosan (CS), erythromycin (ERI).

These results suggest that the decreased MIC value obtained in the case of Fe_3O_4/SA/CS/NEO is obtained by the cumulative effect of Fe_3O_4, SA and

neomycin, while the addition of chitosan to this nanosystem seem to diminish the antimicrobial effect. However, the antimicrobial effect of the nanosystem functionalized with chitosan is still very efficient and the biocompatibility may be also enhanced by using a polymeric coating [11]. These results are supported by recent studies which demonstrate that chitosan coated nanoparticles reduce the citotoxicity of the loaded antimicrobial compound and in the same time ensure an efficient antimicrobial effect and a controlled drug release [12].

4. Conclusions

The reported data suggest that chitosan may be an essential factor in dictating the biological activity of nanostructured materials containing functionalized magnetite and may also control de delivery of antimicrobial drugs.

Our results clearly demonstrate that the obtained nanoparticles significantly improve the antimicrobial activity of the antibiotics neomycin and erythromycin by decreasing the required minimum inhibitory concentration to up to 25 folds.

Sulfanilic acid may improve the antimicrobial effect of magnetite nanoparticles but the citotoxicity of the system is not influenced, hence the prepared nanosystems containing magnetite nanoparticles functionalized with sulfanilic acid only, or with sulfanilic acid/chitosan and antibiotics have a very low *in vitro* citotoxicity.

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