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Biocompatible hydrodispersible magnetite nanoparticles used as antibiotic drug carriers

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Abstract

Here we report a newly synthesized vectorizing nanosystem, based on hydrodispersible magnetite nanoparticles (HMNPs) with an average size less than 10 nm, obtained by precipitation of Fe(II) and Fe(III) in basic solution of *p*-aminobenzoic acid (PABA), characterized by high-resolution transmission electron microscopy (HR-TEM), dynamic light scattering (DLS), X-ray diffraction (XRD), differential thermal analysis coupled with thermogravimetric analysis (DTA-TGA) and bioevaluated for cytotoxicity and antibiotic delivery in active forms. The obtained data demonstrate that HMNPs can be used as an efficient drug delivery system, for clinically relevant antimicrobial drugs. HMNPs antimicrobial activity depended on the loaded drug structure and the tested microbial strain, being more efficient against *Pseudomonas aeruginosa*, comparing with the *Escherichia coli* strain. The novel HMNPs demonstrated an acceptable biocompatibility level, being thus a very good candidate for biomedical applications, such as drug delivery or targeting.

Keywords: antibiotic release, Gram-negative, growth inhibition zone diameters, HeLa cells, fluorescence, apoptosis.

Introduction

Due to their excellent properties, magnetite nanoparticles (MNPs) have proved a great potential for diverse applications in nanobiotechnology, such as drug targeting and delivering [1–6], magnetic resonance imaging [7], inhibition of microbial biofilm development [8], optimization of wound dressings [9], stabilization of essential oils [10] and anti-tumor therapy [11, 12].

For biomedical applications, MNPs should fulfill some special requirements, such as: appropriate shape (*e.g.*, spherical), biocompatibility, superparamagnetism, high crystallinity, suitability for large surface areas usage and good dispersion in liquid media [13].

To integrate MNPs into biological systems, surface coatings are necessary to improve their colloidal stability, cytocompatibility, and decrease immunogenicity of MNPs [14–16].

Our recent findings demonstrate that MNPs can improve the antimicrobial activity of different therapeutic agents. Previous results proved that loading kanamycin sulfate into a water dispersible metal oxide nanobio-composite containing chitosan and magnetite nanoparticles improved the delivery of this drug in an active form reducing the minimum inhibitory concentration of kanamycin by two to four folds depending on the tested bacterial strain [4].

Furthermore, Grumezescu *et al.* (2012) reported that newly synthesized water-soluble magnetite nanoparticles protected by chitosan and polyvinyl alcohol significantly improved the antimicrobial activity of gentamicin, ciprofloxacin and cefotaxime against microbial pathogens, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* [6].

Cotar *et al.* (2013) demonstrated that core@shell MNPs based magnetite filled with amoxicillin and kanamycin decreased the MIC against *Escherichia coli*, comparatively with the MIC values of antibiotic solutions [17]. All fabricated nanomaterials exhibited a low cytotoxic effect on eukaryotic cells being thus a good candidate for developing new antimicrobial strategies aiming to potentiate the antimicrobial effect of drugs and controlling their delivery.

P. aeruginosa is an opportunistic pathogen that could cause a broad range spectrum of infections especially in immunocompromised and chronic patients. This highly versatile bacterium exhibits also a great natural and acquired resistance to many regular antibiotics [18]; therefore, pseudomonadal infections are currently difficult to treat. Another normal resident of intestinal microbiota inhabitant that can cause severe infections if appropriate conditions arise is *E. coli* [19].

Pathogenic variants cause intestinal and extraintestinal infections, including gastroenteritis, urinary tract infections,

meningitis, peritonitis, and septicemia. Surveillance data show that resistance in *E. coli* is consistently high for antimicrobial agents that have been in use for a long time in human and veterinary medicine. The most frequent co-resistant phenotype recently observed in *E. coli* was to tetracycline and streptomycin, followed by tetracycline and sulfonamides [20].

Since we are facing an increasing general microbial resistance and persistence, a recent interest among microbiologists is to develop alternative efficient antimicrobial strategies. Creating novel antimicrobial drugs may be considered the best approach, but this is usually a long-term labor, time and material consuming, and newly developed drugs are usually active only against limited taxonomical groups. Therefore, a more desirable strategy involves maximizing the efficiency of current antibiotics, by using them in low amounts, controlling their release and limiting their action at the site of infection.

In this study, we synthesized and characterized hydrodispersible magnetite nanoparticles (HMNPs) as potential drug carriers for targeted delivery. To determine the physical characteristics of HMNPs, size distribution was analyzed by dynamic light scattering (DLS) and the presence of magnetite was confirmed by X-ray diffraction (XRD). The nanometric size of HMNPs was established by high-resolution transmission electron microscopy (HR-TEM). Loading a coating of organic shell on the surface of HMNPs was analyzed using differential thermal analysis coupled with thermogravimetric analysis (DTA–TGA). *In vitro* biological assays demonstrated that HMNPs can be a highly efficient and biocompatible drug carrier useful for the improvement of classical antimicrobial therapies.

Materials and Methods

Synthesis of HMNPs

Hydrodispersible magnetite nanoparticles (HMNPs) were prepared by a modified co-precipitation method [21]. One gram of *p*-aminobenzoic acid (PABA) was solubilized in a known volume of ultrapure water, corresponding to a 1.00% (*w/w*) solution, under stirring at room temperature. Then, 8 mL of a basic aqueous solution consisting of 25% ammonia were added to PABA solution. After these, 100 mL aqueous solution of $FeCl_3$ and $FeSO_4 \cdot 7H_2O$ (molar ratio 2/1) were dropped under permanent stirring up to pH 8. The product was washed using ultrapure water and methanol and separated with a strong NdFeB permanent magnet.

Characterization of HMNPs

X-ray diffraction (XRD)

X-ray diffraction (XRD) 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 $10–80^0$.

Transmission electron microscopy (TEM)

The transmission electron microscopy (TEM) images were obtained on samples using a Tecnai™ G2 F30 S-TWIN high-resolution transmission electron microscope from FEI Company (OR, USA). The microscope operated in transmission mode at 300 kV with TEM point resolution of 2 Å and line resolution of 1 Å.

Differential thermal analysis coupled with thermogravimetric analysis (DTA–TGA)

The differential thermal analysis (DTA) coupled with thermogravimetric analysis (TGA) was performed with a Shimadzu DTG-TA-50H, at a scan rate of 10^0C/min., in air.

Dynamic light scattering (DLS)

Particles size analysis was performed using intensity distribution by dynamic light scattering technique (Zetasizer Nano ZS, Malvern Instruments Ltd., UK), at scattering angles of 90^0 and 25^0. The average diameters (based on Stokes–Einstein equation) were calculated from three individual measurements. The *zeta* potential was measured using the Zetasizer Nano ZS.

Antimicrobial activity assay

An adapted diffusion method was used in order to assess the influence of the water soluble nanovehicle on the antimicrobial activity of piperacillin–tazobactam (TZP), cefepime (FEP), piperacillin (PIP), imipenem (IPM), gentamicin (CN), ceftazidime (CAZ) against *P. aeruginosa* ATCC 27853 strain and cefazolin (KZ), cefaclor (CEC), cefuroxime (CXM), ceftriaxone (CRO), cefoxitin (FOX), trimethoprim–sulfamethoxazole (SXT) against *E. coli* ATCC 25922 strain. Bacterial strains were purchased from ATCC (American Type Culture Collection, US). The tested antibiotics have been chosen according to Clinical and Laboratory Standards Institute (CLSI) recommendations, and purchased from Oxoid (UK). Standardized antibiotic discs have been placed on the Müller–Hinton agar (Oxoid, UK) medium distributed in Petri dishes seeded with a bacterial inoculum with an optical density corresponding to the 0.5 McFarland standard (~10^8 CFU/mL). Five µL of the stock solutions of the water-soluble nanovehicle were spotted over the antibiotic disks. The plates were incubated for 24 hours at 37^0C, and the inhibition zones diameters for each antibiotic, after the addition of the tested nanomaterial solution was quantified and compared with the growth inhibition zones obtained for the respective antibiotics in solution.

Biocompatibility

Fluorescence microscopy

5×10^5 HeLa cells were seeded in each well of a 24-well plate (Nunc) in 10% fetal bovine serum (FBS) supplemented Dulbecco's Modified Eagle's medium (DMEM, Sigma Aldrich). When cell monolayer reached 70–80% confluence (after about 24 hours), the cells were treated with HMNPs in the final concentration 100 µg/mL, 500 µg/mL. The effect of HMNPs was evaluated after 24 hours by adding 100 µL of propidium iodide (0.1 mg/mL) (PI) and 100 µL fluorescein diacetate (FdA). Fluorescence was quantified using Observer.D1 Carl Zeiss microscope (Zeiss).

Apoptosis detection

Flow cytometry analysis was performed to discriminate between intact and apoptotic cells using fluorescein isothiocyanate (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 5 µL Annexin V-FITC and 5 µL propidium iodide for 10 minutes in dark chamber. For each sample, at least 10 000 events were acquired using a Beckman Coulter flow cytometer (BD) and results were analyzed using FlowJo software.

Results

The purity and crystalline properties of the HMNPs was investigated by XRD. The XRD pattern is shown in Figure 1. The diffraction peaks centered at $2\theta = 30.32^0$, 35.72^0, 43.42^0, 57.44^0 and 63.08^0 indicate the formation of well-crystallized Fe_3O_4 [22].

The content of PABA was estimated based on the characteristic weight losses of the HMNPs (Fe_3O_4@PABA) and pure MNPs (Fe_3O_4) sample (Figure 2). The content of water in HMNPs is ~5.06% (weight loss between room temperature – RT and 140^0C). When compared with pure MNPs (water content ~4.11%), those coated with PABA shell induce a slight higher water retention, which is explained based on the presence of free amino groups. The content of PABA is determined based on the weight loss up to 1000^0C and was estimated to be 2.02%. Based on these, the content of magnetite was estimated to be 92.92%. Water loss is accompanied by an endothermic effect, while PABA degradation is associated with a strong exothermic peak, centered at 558^0C.

Newly synthesized HMNPs exhibited a positive *zeta* potential of about 70 mV (image not shown). This value is favorable for the electrostatic interaction with the negatively charged bacterial wall [23]. The positive surface charge is provided by the amino groups of PABA. In general, it is considered that the nanoparticles displaying a positive surface charge are internalized into cells more efficiently than the nanoparticles with a negative surface charge [24, 25].

The average hydrodynamic particle size of HMNPs at 25^0C observed in DLS (Figure 3) is about 9 nm. The results are in good agreement with TEM analysis (Figure 4). According to Figure 4a, the HMNPs have a spherical shape ranging from 4 to 9 nm. The crystalline nature of HMNPs is also confirmed by selected area electron diffraction (SAED) (Figure 4b).

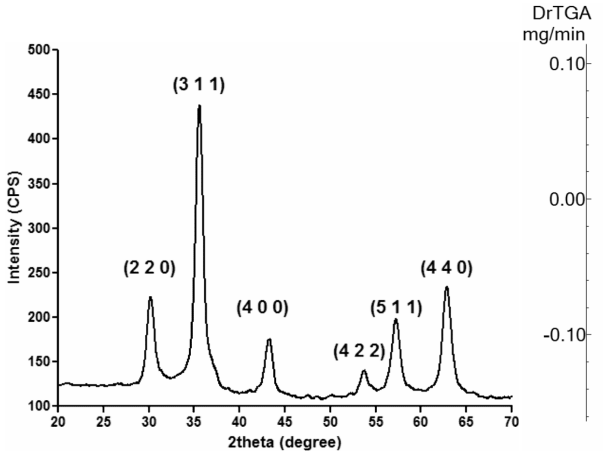

Figure 1 – ***XRD pattern of HMNPs.***

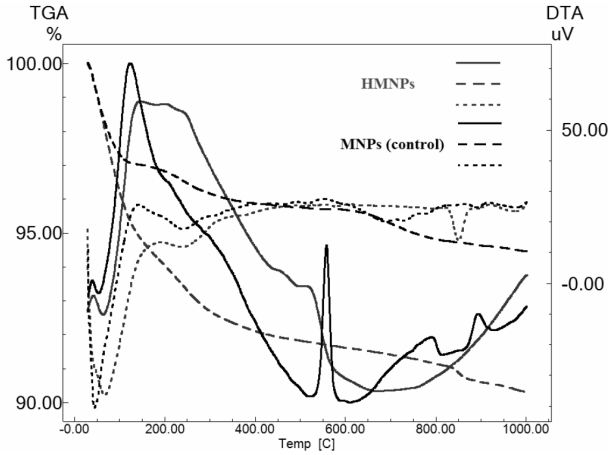

Figure 2 – ***DTA–TGA of HMNPs.***

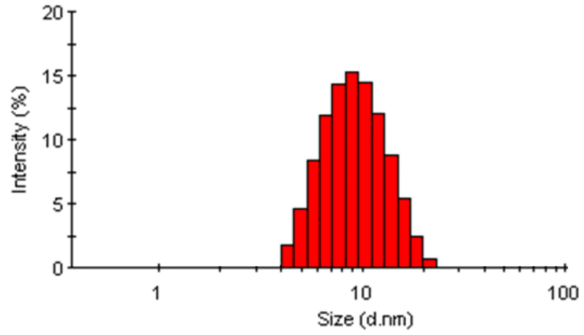

Figure 3 – ***Size distribution histogram of HMNPs.***

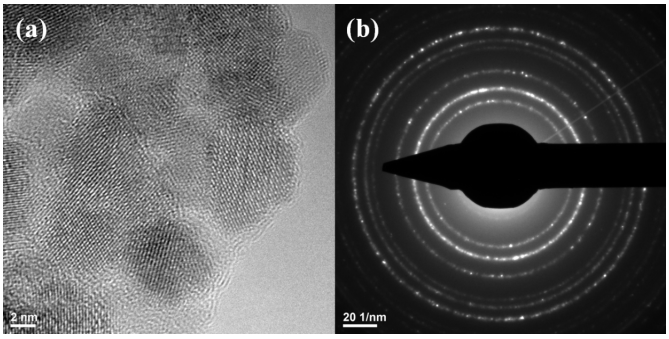

Figure 4 – ***HR-TEM image (a) and SAED pattern (b) of HMNPs.***

Cytotoxicity assays revealed that HMNPs have a very low toxic effect on eukaryotic cells. Microscopy testing demonstrate that the tested concentrations of 100 µg/mL or 500 µg/mL did not induce significant changes in HeLA cells morphology (Figure 5), the cells remaining normally attached on the bottom of the well and exhibiting a normal morphology.

Microscopy results were confirmed by the flow cytometry assay, revealing a very low percentage of dead *vs.* live cells (Figure 5). Flow cytometry revealed that when used at 100 µg/mL, HMNPs have no significant effect on HeLA cells apoptosis or necrosis, while in higher amounts (500 µg/mL) induced only a slight apoptosis of the eukaryotic cells. However, at both tested concentrations, the viability of HeLA cells was maintained at a high percentage of at least 90% (Figure 6).

Antimicrobial activity assays demonstrated that the obtained HMNPs improved the efficiency of loaded anti-

biotics comparing with antibiotic solutions, as revealed by the increase in growth inhibition zone diameter observed for all tested antibiotics. The most significant potentiating effect was achieved for IPM (more than two-fold greater comparing with IPM control), PIP and FEP in case of *P. aeruginosa* (Figure 7).

Antibiotic susceptibility assay in *E. coli* revealed a weaker synergy of the HMNPs with the screened antibiotics, comparing with *P. aeruginosa*, an enhanced efficiency being observed only for FOX, all other tested antibiotics revealing similar inhibition zones when used in solution or HMNP-bound (Figure 8).

The antimicrobial testing results revealed that HMNPs could enhance specifically the antibiotics activity, depending on the antibiotic structure, and also on the tested bacterial strain.

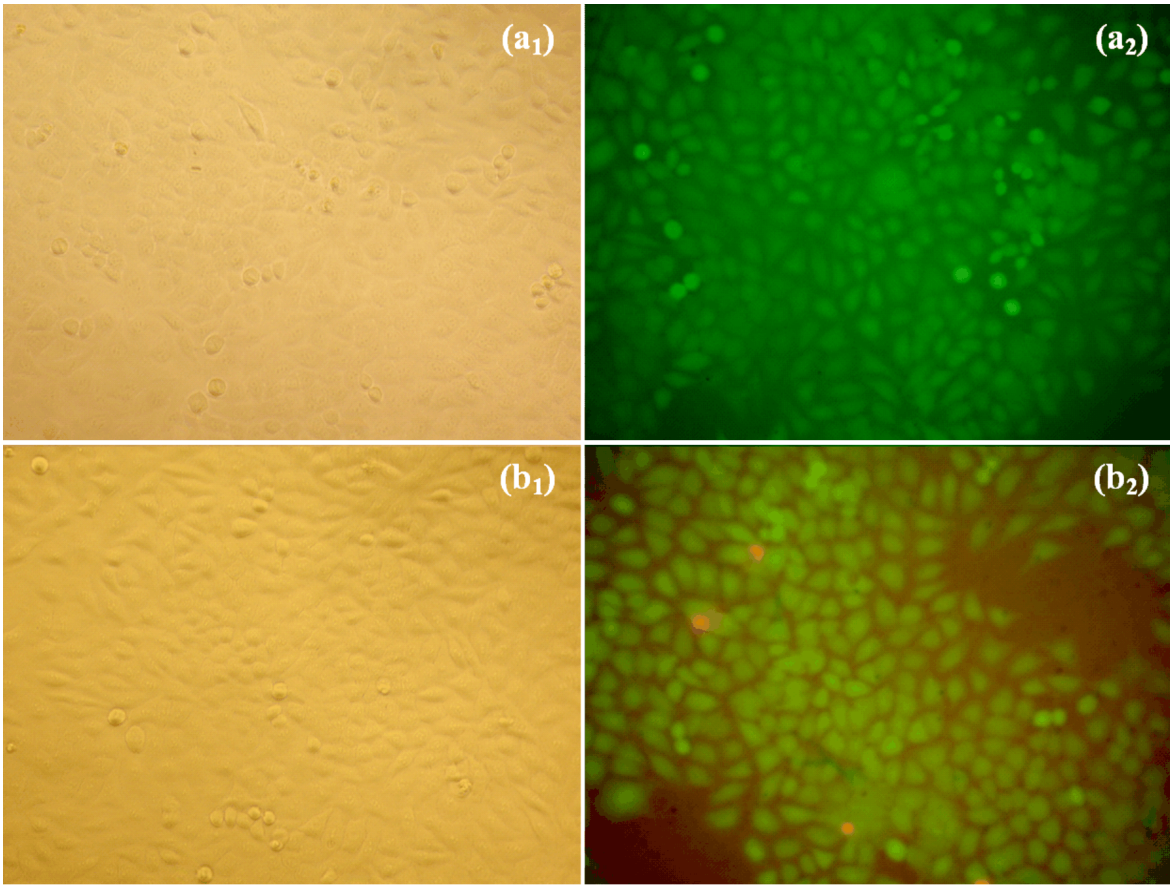

Figure 5 – ***The effects of different concentration of HMNPs on HeLa cells: 100 µg/mL HMNPs (a_1 – transmission, a_2 – fluorescence); 500 µg/mL HMNPs (b_1 – transmission, b_2 – fluorescence).***

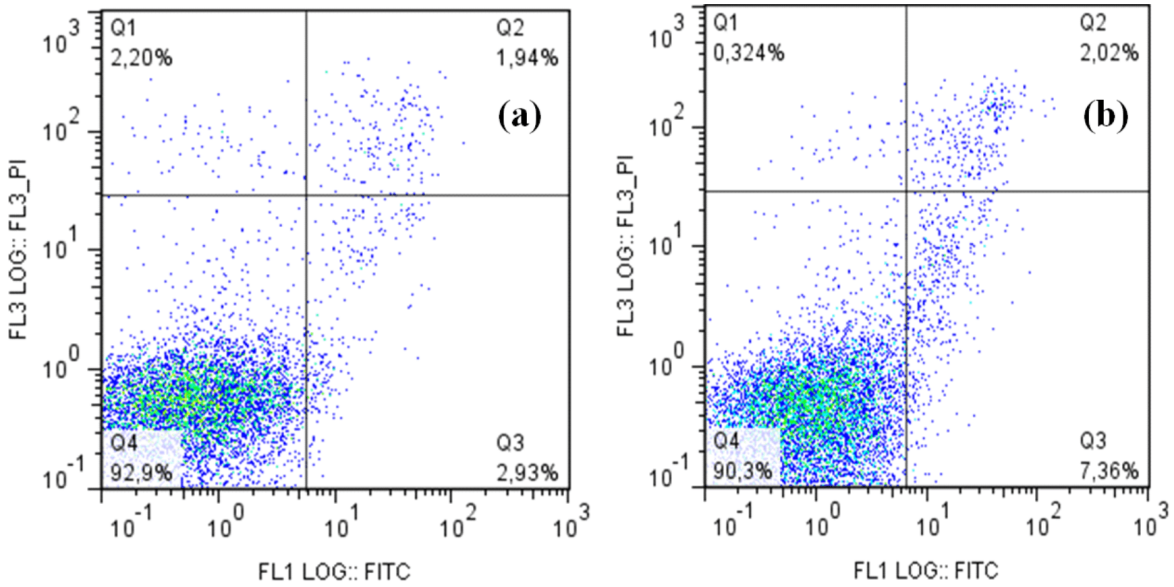

Figure 6 – ***Quantifying HeLa apoptosis and necrosis rates after 24 h treatment with 100 µg/mL (a) and 500 µg/mL (b) of HMNPs. Apoptotic cells (FITC+/PI-, green fluorescence), necrotic or late apoptotic cells (FITC+/PI+, red and green fluorescence), and viable cells (FITC-/PI-, non fluorescent).***

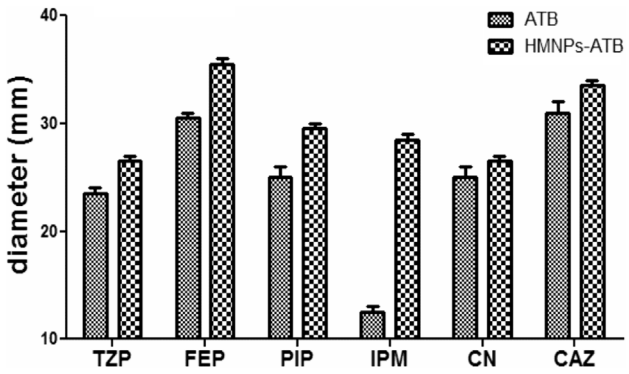

Figure 7 – ***Comparison of growth inhibition diameters induced by HMNP-bound antibiotics vs. antibiotics solutions on P. aeruginosa.***

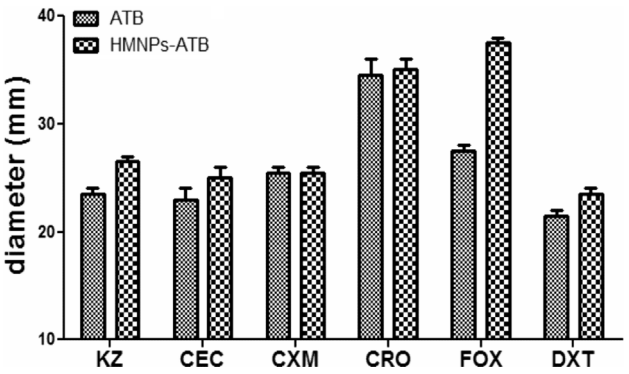

Figure 8 – ***Comparison of growth inhibition diameters induced by HMNP-bound antibiotics vs. antibiotics solutions on E. coli.***

Discussion

Infectious diseases remain a major world health problem due to the rapid development of resistance to the existing antimicrobial drugs among resistant Gram-positive and Gram-negative bacteria and fungal strains [26, 27]. This problem is even more threatening due to the very limited number of new antimicrobial agents that are in development [28]. The need for new drugs is critical for some resistant Gram-negative strains, such as *P. aeruginosa* and *Enterobacteriaceae*.

Nanotechnology could represent an important option for the development of new and efficient antimicrobial strategies, one of them being that approached in the present study, of improving the activity of the current of the current antibiotics [29]. The incorporation of antibiotics into nanoparticles facilitates a better penetration into the tissues, the drugs could be released with a controlled and predetermined rate, for a sufficient period of time to reach the target site, that could significantly increase the therapeutic index and the efficacy of treatment and reduce the harmful side effects on the target organ [30].

The purpose of this study was to investigate the ability of magnetite nanoparticles to improve the activity of antibiotics against Gram-negative bacteria, which are known for their multi-drug, extended-drug and even pan-drug resistance to current antibiotics, being responsible of severe and hard to treat infections.

Although iron is not considered a conventional antimicrobial agent, being on the contrary, required by the microbial cells for gene activation and different physiological processes, however the antimicrobial effect of iron oxide nanoparticles has been shown in many studies and attributed to their ability to cross the bacterial wall, to interfere with the cellular membrane functions and to induce the production of reactive oxygen species [29].

The obtained results are encouraging, as for many antibiotics belonging to classes largely used in clinics (antipseudomonal penicillins, penicillins + beta-lactamase inhibitors, second, third and fourth generation cephalosporins, carbapenems), but unfortunately inactivated by different enzymes (*i.e.*, extended spectrum beta-lactamases, carbapenemases, etc.), an important improvement of their activity was obtained in the presence of the magnetite nanoparticles carrier.

Conclusions

The obtained results revealed that antibiotics bound to HMNPs exhibit a more intensive growth inhibition effect on versatile pathogens, as *P. aeruginosa* and *E. coli*, in a specific manner, depending on the tested bacterial strain and the drug structure. The HMNPs are thus good candidates for biomedical applications, such as drug delivery and controlled release nanosystems, exhibiting also the advantages of a very easy synthesis method and low cytotoxicity rates.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

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