ISSN 2284 6808

Open Access Journal

Letters in Applied NanoBioScience

Volume 2, Issue 1, 97-104, 2013

www.NanoBioLetters.com

NANOTECHNOLOGICAL SOLUTION FOR IMPROVING THE ANTIBIOTIC EFFICIENCY AGAINST BIOFILMS DEVELOPED BY GRAM-NEGATIVE BACTERIAL STRAINS

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Article info

Received: 12.01.2013
Accepted: 10.03.2013
Published: 30.03.2013

Abstract

At present bacteria involved in biofilm associated infections display the highest rates of antibiotic resistance among pathogenic bacteria, which made that treatment options to be limited, and determined the researchers to find out alternative treatments to antibiotics. In the recent years nanomaterials gained much attention in medicine, particularly in the fight to bacteria resistant to antibiotics by acting as drug delivery devices. Magnetic iron oxide nanoparticles (MNPs) have raised much interest during the recent years due to their potential applications in medicine. In the present study we synthesized MNPd functionalized with antibiotics for the study of their antimicrobial and anti-biofilm properties against Escherichia coli and Pseudomonas aeruginosa, two Gram-negative bacteria, frequently resistant to antibiotics, involved in biofilm infections in order to investigate their capacity to serve as potential drug delivery systems in the fight to these important opportunist pathogens.

Keywords *Multidrug resistance, biofilms, magnetite nanoparticles, drug delivery systems*

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Introduction

Biofilms are estimated to be involved in around 80% of all chronic human infections [1], around half of them being related to the use of an indwelling medical device [2]. With many millions of medical devices being used each year [3], biofilms constitute a significant public health risk for patients requiring such devices [4]. Also, it has been estimated that 65% of nosocomial infections are biofilm associated, loading the health care system enormous costs [5]. Among Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa* are the most frequently involved in infections due to biofilm formation. A worrying feature of biofilm-based infections is represented by their higher resistance to antibiotics and disinfecting chemicals as well as to phagocytosis and other components of the body's defence system, when compared to planktonic cells [6]. *Escherichia coli* is an important pathogen causing 80 to 90% of community-acquired urinary tract infections (UTIs) and more than 30% of nosocomially acquired UTIs [7-9]. Clinical observations have established that the microbial populations within catheter associated UTI frequently develop in biofilms, directly attaching to the surface of catheters [5]. Biofilm can promote persistence in the urinary tract and on biomaterial surfaces by protecting bacteria from the clearing out effect of hydrodynamic

forces and the killing activity of host defence mechanisms and antibiotics [10].

Ps. aeruginosa is an effective opportunistic pathogen responsible for chronic lung infection in cystic fibrosis patients, nosocomial infections in immuno-compromised patients [11, 12], and severe burns to those who are in contact with contaminated medical devices [13]. Unfortunately, some of nosocomial infections are frequently life threatening and often challenging to treat; especially with the frequent emergence of resistance to multiple drugs in this pathogen [14]. This resistance can emerges gradually during exposure to antipseudomonal antibiotics [15], this emergence was reported in 27–72% of patients with initially susceptible *Ps. aeruginosa* isolates and usually results in higher morbidity, mortality and economic burden [14]. As biofilm infections significantly contribute to patient morbidity and substantial healthcare costs, novel strategies to treat these infections are urgently required. In the recent years nanomaterials gained much attention in medicine, particularly in the fight to bacteria resistant to antibiotics by acting as drug delivery devices [16]. The synthesis of multifunctional magnetic nanoparticles (NPs) is a highly active area of current research located at the interface between materials science, biotechnology and medicine. Magnetic nanoparticles (MNPs), fabricated by loading a therapeutic agent into a magnetic nanoparticle through encapsulation or adsorption, have gained particular interest during the last decade because of their intrinsic magnetic nature as well as enhanced physicochemical properties [17]. MNPs represent a subclass within the overall category of nanomaterials and are widely used in many applications, particularly in the biomedical sciences such as targeted delivery of drugs or genes, in magnetic resonance imaging, and in hyperthermia (treating tumors with heat) [18]. Using their superior specifications MNPs can address the shortcomings of traditional therapeutic agents especially antimicrobials [17]. MNPs show great potential as carriers for targeted drug and gene delivery, since reactive agents, such as drug molecules or large biomolecules (including genes and antibodies), can easily be attached to their surface [10]. On the other hand, the fate of the nanoparticles inside the body is mainly determined by the interactions with its local environment. These interactions strongly depend on the size of the magnetic NPs, but also on the individual surface characteristics, like charge, morphology and surface chemistry [19]. Iron oxide nanoparticles in combination of external magnetic field allows delivering particles to the desired target area and fixing them at the local site while the pharmaceutical drug is released and acts locally [20-22]. 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 [23].

There are important studies that have shown that different nanoparticles exhibited antimicrobial activity against pathogenic bacteria [24]. In a study the researchers have demonstrated that ZnO, CuO, and Fe_2O_3 nanoparticles possess antimicrobial activity against Gram-negative and Gram-positive bacteria [25].

In this study we have investigated the efficiency of magnetic nanoparticles functionalized with antibiotics as potential inhibitors of growth and biofilm formation by two important Gram-negative bacteria involved in biofilm formation infections, difficult to treat due to their antibiotic resistance.

Experiment Details

Preparation of magnetite nanoparticles functionalized with antibiotics. All chemicals were used as received. $FeCl_3$, $FeSO_4 \cdot 7H_2O$, NH_4OH (25%), and CH_3OH were purchased from Sigma-Aldrich ChemieGmbh (Munich, Germany)**.** Magnetic iron oxide nanoparticles (MNPs) were prepared by wet chemical precipitation from aqueous iron salt solutions by means of alkaline media, like HO^-, NH_3 [27,28]. Briefly, $FeSO_4x7H_2O$ and $FeCl_3$ (1/2 molar ratio) was dissolved in 200 mL of ultrapure water in a beaker. Then 4 mL of NH_4OH (25%) and 100 mg ATB were dissolved in 300 mL of ultrapure water. Under stirring,

the above Fe(II)/Fe(III) solution was added to the NH_4OH /ATB solution in the beaker. A black precipitate formed immediately. The resultant solid was washed with ultrapure water three times and then finally with methanol. After washing, prepared MNPs@ATBs were dried at room temperature.

Characterization of the obtained nanostructure. 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 15 mA and 30 kV) was used. The samples were scanned in the Bragg angle 2θ range of 10-80.

TG Analysis. The thermogravimetric (TG) analysis of the biocomposite was assessed with a Shimadzu DTG-TA-50H instrument. Samples were screened to 200 mesh prior to analysis, were placed in alumina crucible, and heated with 10 $K \cdot min^{-1}$ from room temperature to 800°C, under the flow of 20 $mL \cdot min^{-1}$ dried synthetic air (80% N_2 and 20% O_2).

DLS. Particles size were determined by using dynamic light scattering technique (Zetasizer Nano ZS, Malvern Instruments Ltd., U.K.), at a scattering angle of 90^o and 25^oC. The particle size analysis data was evaluated using intensity distribution. The average diameters (based on Stokes-Einstein equation) were calculated from the three individual measurements.

Assessment of the influence of magnetic nanoparticles functionalized with antibiotics on planktonic cells growth (minimal inhibitory concentration –MIC assay) and biofilm development (microtiter method). The study of antimicrobial activity of magnetic nanoparticles functionalized with antibiotics against two reference bacterial strains: *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 was performed by a quantitative method for MIC (minimal inhibitory concentration) determination of each naonosystem magnetite/antibiotic. This method consist of two-fold microdilutions of nanoparticules stock solutions prepared in sterile saline performed in liquid culture medium (nutrient broth) distributed in 96 multi-well plates. For these experiments the following antibiotics were used: cefotaxime (CTX), polymyxin B (PB) for both *E. coli* and *Ps. aeruginosa*, to which amoxicillin (AMX), kanamycin (K) and streptomycin (S), were added for *E. coli*. Each well was inoculated with 5 μL of microbial suspensions of 0.5 Mac Farland density. Sterility control wells (nutrient broth) and microbial growth controls (inoculated nutrient broth) were used. The plates were incubated for 24 h at 35 ±2°C, and the influence of nanoparticles on the planktonic cells growth in the liquid medium was appreciated by measuring the absorbance at 600 nm of the obtained cultures. The MIC for each Fe_3O_4@ABT and each antibiotic, respectively, was considered as the last dilution of the tested compound which inhibited the microbial growth. Afterwards, the 96 well plates were emptied, washed 3 times with phosphate buffered saline, fixed with cold methanol and stained with violet crystal solution 1% for 30 min. The biofilm formed onto the plastic wells was resuspended in 30% acetic acid and the intensity of the stained suspension was assayed by measuring the absorbance at 490 nm [29]. The results interpretation was performed by comparing the obtained value of absorbance at 490 nm for strains treated with nanoparticles with those obtained for control strains (bacteria grown in standard/normal conditions).

Results and Discussions

XRD patterns (Figure 1) shows that the MNPs are well-crystalline and exhibit diffraction peaks at 30.5^o(220), 35.9^o(311), 37^o(222), 43.5^o(400), 57.3^o(511) and 63.1^o(440), which match the standard pattern of Fe_3O_4 planes of cubic crystal system (JCPDS file No. 19-0629). The amount of ATB (CTX, PB, AMX, K) from MNPs@ATB was estabilished by TG analysis (figure 2). The wight lost of ATB amount from Fe_3O_4@ATB varies between 1 and 4 % as follow: MNPs@PB (1,52%), MNPs@CTX (2.46), MNPs@S (2.76%), MNPs@K (3.84%). The mean hydrodynamic size of the synthesized samples are plotted in Figures

3-6. As is seen from these figures and mean hydrodynamic size is between 56 and 78 nm.

Two fold serial microdilution method in broth medium allowed us to establish the MIC of nanoparticles functionalized with each antibiotic against the two reference strains of Gram-negative bacteria.

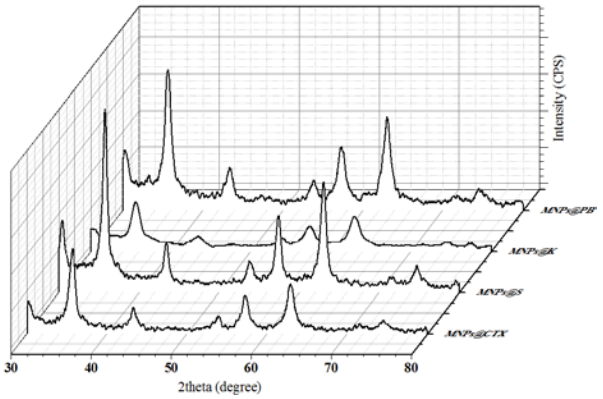

Figure 1: *XRD patterns of MNPs@PB, MNPs@K, MNPs@S, MNPs@CTX*

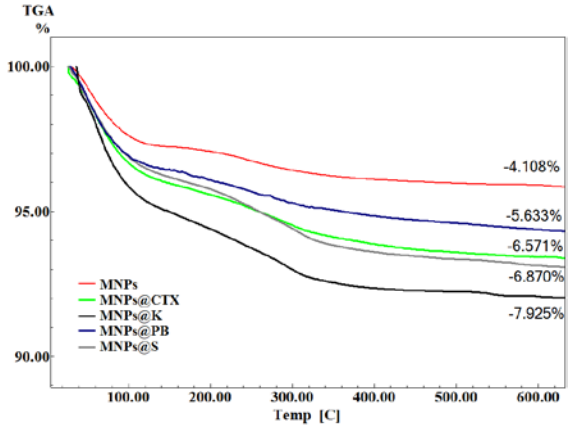

Figure 2: *TG analysis of MNPs@PB, MNPs@K, MNPs@S, MNPs@CTX*

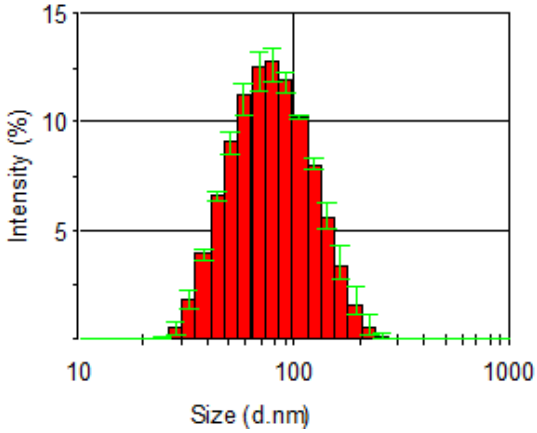

Figure 3: *Size distribution of MNPs@CTX evaluated by DLS technique: 72 nm*

The obtained results were showing a decrease of the MIC value of magnetite nanoparticles functionalized with the antibiotics AMX and K against *E. coli* comparatively with the MIC values of antibiotics solutions. In the case of *Ps. aeruginosa*, the MIC values of Fe_3O_4@CTX decreased at 5.85 µg/mL comparatively with the MIC value of 8 µg/ml of the antibiotic solution against *Ps. aeruginosa* reference strain. The results concerning the effect expressed by nanoparticles functionalized with antibiotics on biofilm formation of analyzed strains showed their inhibitory activity on both analyzed bacteria.

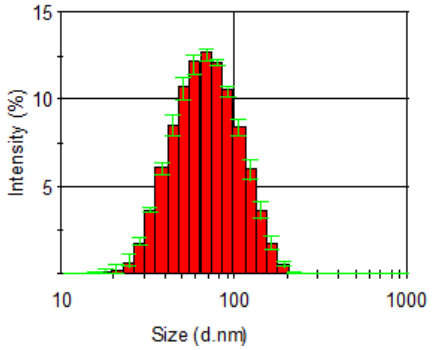

Figure 4: *Size distribution of MNPs@PB evaluated by DLS technique: 62 nm*

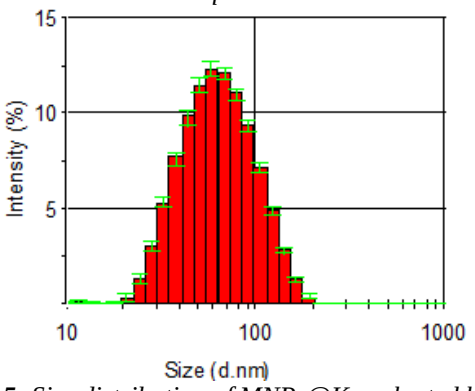

Figure 5: *Size distribution of MNPs@K evaluated by DLS technique: 56 nm*

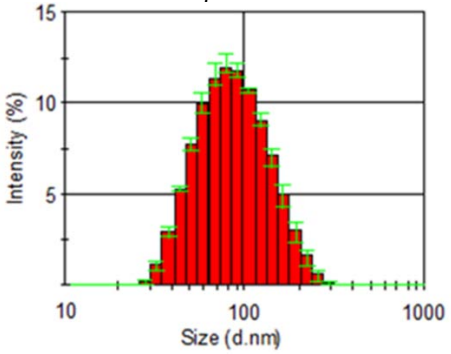

Figure 6: *Size distribution of MNPs@S evaluated by DLS technique: 78 nm*

The magnetite functionalized with kanamycin exhibited the highest inhibitory activity against biofilm formation by *E. coli* strain, being followed by those functionalized with polymixin B, cefotaxime and streptomycin (Fig. 7-10).

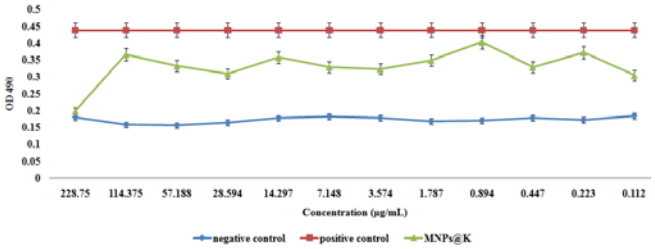

Figure 7: *The effect of MNPs@K on biofilm formation in E. coli*

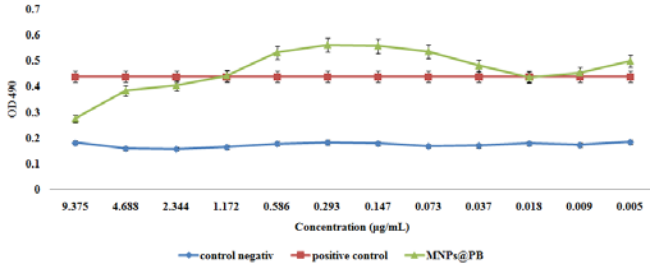

Figure 8: *The effect of MNPs@PB on biofilm formation in E. coli*

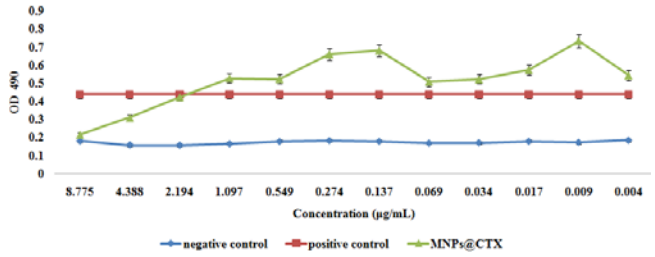

Figure 9: *The effect of MNPs@CTX on biofilm formation in E. coli*

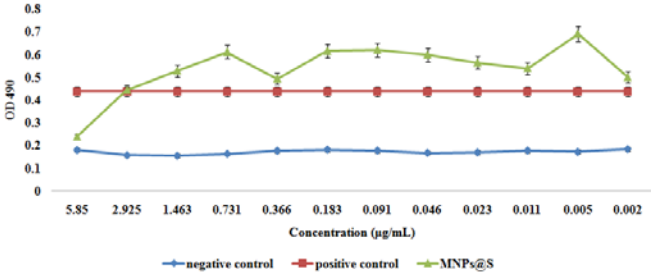

Figure 10: *The effect of MNPs@S on biofilm formation in E. coli*

In case of *Ps. aeruginosa* MNPs@PB exhibited a higher inhibitory activity on biofilm formation by *Ps. aeruginosa* reference strains than MNPs@CTX (Figure 11, 12). Also, we observed that the inhibitory activity of the nanoparticles were dependent of the antibiotic concentrations. Thus, the inhibitory activity of magnetite functionalized with polymixin B and cefotaxime on biofilm formation by *E. coli* was exhibited from the first until the third concentration, while for magnetite functionalized with streptomycin it inhibitory effect was mantained until the second concentration (Fig. 8-10). The inhibitory activity of MNPs@PB of biofilm formation by *Ps. aeruginosa* strain was maintend until the fourth concentration, while the inhibitory activity of MNPs@CTX was expressed until the second concentration of antibiotic used (Figure 11, 12).

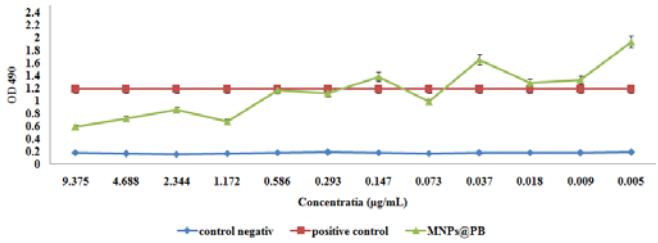

Figure 11: *The effect of MNPs@PB on biofilm formation in Ps. aeruginosa*

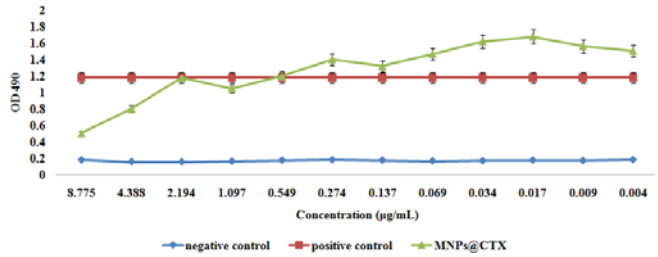

Figure 12: *The effect of MNPs@CTX on biofilm formation in Ps. aeruginosa*

At present we are facing with the increasing number of studies concerning the finding out of new alternative antimicrobial strategies to fight to biofilm associated infections, such as urinary tract infections with *E. coli*, or cystic fibrosis with *Ps. aeruginosa*. One strategy which gained much attention in the recent years is based on the synthesis of nanoparticles with antimicrobial activity or which can be used as drug delivery systems. There are a lot of reports on the antimicrobial and anti-biofilm properties of different types of nanoparticles, especially metals or metallic oxide-containing ones (silver, copper, gold, and ZnO) [30-34], as well as core/shell nanosystems (e.g., $CoFe_2O_4$/oleic acid, Fe_3O_4/oleic acid, and Fe_3O_4/PEG_{600}) [35-38] that could be manipulated and

improved, potentially providing a new method for treating antibiotic-resistant device related infections [39, 40]. Nano-silver coatings have been applied to several medical devices, of which catheters, drains, and wound dressings are the most prominent [41]. In other study the research team used Fe_3O_4/C_{12} nanoparticles with 2-((4-ethylphenoxy) methyl)-N-(substituted-phenylcarbamothioyl)-benzamides for obtaining functionalized catheter surfaces. The obtained results have shown improved resistance to *in vitro* microbial colonization and biofilm development by *Ps. aeruginosa* ATCC 27853. The obtained nanofluids proved to be not cytotoxic and did not influence the eukaryotic cell cycle. The long-lasting efficacy of compounds loaded on nanoparticles could be regarded as a future solution to provide persistent, broad-spectrum antibacterial effects with minimal side effects [40]. Silver nanoparticles (AgNPs) have emerged as a potential alternative to conventional antibiotics because of their potent antimicrobial properties [42]. The authors of an interesting study showed that silver nanoparticles (AgNP) were intrinsically antibacterial, whereas gold nanoparticles (AuNP) were antimicrobial only when ampicillin was bound to their surfaces. Both AuNP and AgNP functionalized with ampicillin were effective broad-spectrum bactericides against Gram-negative and Gram-positive bacteria. Most importantly, when AuNP and AgNP were functionalized with ampicillin they became potent bactericidal agents with unique properties that subverted antibiotic resistance mechanisms of multiple-drug-resistant bacteria [43].

The present study has shown that MNPs functionalized with antibiotics displayed inhibitory activity on growth and biofilm formation of *Ps. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922, properties that can be exploited in future studies aiming to investigate their effect on strains isolated from infections associated with biofilm formation in order to study their potential use as drug delivery systems.

Because of their potent antimicrobial activity and unique mode of action, nanoparticles could thus offer an attractive alternative to conventional antibiotics in the development of new-generation antibiotics due to their potent antimicrobial activity and unique mode of action [26].

Conclusions

As biofilm infections significantly contribute to patient morbidity and substantial healthcare costs, novel strategies to treat these infections are urgently required. The use of nanoparticles is a growing new approach against biofilm-mediated, drug-resistant, and device centered infections. The obtained results concerning the antimicrobial activity and ability to inhibit biofilm formation of MNPs functionalized with antibiotics are suggesting that these can be used in the future experiments for demonstration their efficacy on pathogenic strains, especially on *E. coli* in order to validate the results obtained on reference strain. Future studies must concentrate on studying the potential use of these nanosystems for the treatment of catheter associated urinary tract infections caused by *E. coli* strains.

Acknowledgments

The results presented in this work were supported by the strategic grant POSDRU/89/1.5/S/58852, Project "Postdoctoral program for training scientific researchers" cofinanced by the European Social Fund within the Sectorial Operational Program Human Resources Development 2007–2013", Human Resources Project no 135/2010 (Contract no. 76/2010) and Ideas contract no. 154/05.10.2011.

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