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Water dispersible magnetite nanoparticles influence the efficacy of antibiotics against planktonic and biofilm embedded *Enterococcus faecalis* cells

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ABSTRACT

The objective of this study was to investigate the potential of magnetic nanoparticles to potentiate, but also to accomplish a sustained and controlled drug release and subsequently improve the efficacy of antibiotics against *Enterococcus faecalis*, one of the most resistant opportunistic pathogens, that poses a threat to chronically infected or immunocompromised patients and is difficult to eradicate from medical devices. To our knowledge, this is the first study trying to investigate the ability of magnetite nanoparticles to improve the anti-bacterial activity of the current antibiotics against planktonic and biofilm growing *E. faecalis*. Our results are suggesting that the magnetite nanoparticles may be considered an effective aminoglycoside antibiotics carrier, but a complete understanding of the way in which they selectively interact with different antibiotics and with the bacterial cell is needed, in order to obtain improved strategies for elimination of *E. faecalis* biofilms on biomedical devices or human tissues.
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1. Introduction

Although enterococci are natural inhabitants of the oral cavity, normal intestinal microflora, and female genital tract of both human and animals, they could infect the urinary tract, bloodstream, intra-abdominal and pelvic regions, surgical sites and central nervous system [1]. *Enterococcus faecalis* is responsible for 80–90% of human enterococcal infections [2], being one of the most important nosocomial opportunistic pathogens. Hospital-acquired infections caused by enterococci have increased dramatically since the 1970s, accounting for approximately 7%–10% of all bloodstream infections in Europe and respectively in US intensive care units [3,4]. This Gram positive bacterium often infects root canals during endodontic dental treatments of patients with persistent apical periodontitis [5]. Among antibiotic selection pressure, other risk factors should be considered for the emergence of enterococci in hospitals, one of them being their ability to form biofilms on medical devices [1]. This putative virulence factor is responsible for the increasing proportion of enterococcal bacteraemias associated with central venous catheters [1,6]. Clinical enterococcal strains have been shown to adhere to biomedical polymers *in vitro*, but the intimate mechanisms and the clinical relevance of this finding have not been determined yet [1]. These bacteria are considered at present the most antibiotic- and heat-resistant pathogens [7], which determined the intensification of studies for finding out new alternatives to antibiotic treatment.

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 [8]. Metallic nanoparticles may be promising agents for antibacterial applications, due to their

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selective mechanism of toxicity, subsequent to binding to bacterial cell walls and causing membrane disruption through direct interactions or through free radical production [9]. Moreover, phagocytic cells are able to internalize nanoparticles, and to degrade them by lysozomal fusion, reducing host tissues toxicity and free radical damage [10].

Previous studies have demonstrated that ZnO, CuO, and Fe_2O_3 nanoparticles possess antimicrobial activity against Gram-positive and Gram-negative bacteria [9,11,12].

The metallic nanoparticles have been studied for the design of nanotextured surfaces that can reduce microbial adhesion, proliferation, and biofilm growth through emergent antimicrobial properties [4,13–15]. Beside the improvement of tissue-forming cell functions, that nanofeatured medical device surfaces could simultaneously prevent bacterial colonization by enhancing surface energy, increasing selective protein adsorption and promoting protein bioactivity [4,16,17]. This idea has been already used in industry, as antimicrobial nanoparticles are incorporated into numerous paints and other materials that affect our daily lives [4].

Despite the efficacy of nanocoatings to prevent biofilm formation on catheters, there is an important limitation, i.e. the ability of the material to adsorb always the same concentration of the drug and also the ability to control their release, which in most cases results in a non-controlled elution of the drug in the first hours subsequent to the insertion [18–20]. Therefore, the objective of this study was to investigate the potential of magnetic nanoparticles to incorporate antibiotics in an active form, to potentiate, but also to accomplish a sustained and controlled drug release and subsequently to improve the efficacy of different antibiotics against planktonic and biofilm growing *E. faecalis* cells.

2. Materials and methods

2.1. Materials

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).

2.2. Nanostructure fabrication

Magnetic iron oxide nanoparticles were prepared by wet chemical precipitation from aqueous iron salt solutions by means of alkaline media, like NH_3 [21–23]. Briefly, 100 mg of each of the following antibiotics (ATB): vancomycin (VA), penicillin (P), and streptomycin (S), and 8 mL of NH_4OH (25%) were added in 200 mL deionized water under vigorous stirring. Then, 1 g of $FeCl_3$ and 1.6 g of $FeSO_4 \cdot 7H_2O$ were dissolved in 200 mL of deionized water and Fe^{+3}/Fe^{2+} solution was dropped into the basic solution of ATB. After the precipitation of magnetite-antibiotics crystals (Fe_3O_4@ATB), it was repeatedly washed with methanol, separated with a strong NdFeB permanent magnet. Subsequently, the Fe_3O_4@ATB was added into the 100 mL solution of acetic acid 0.1 N and stirred for 10 min. After this, the Fe_3O_4@ATB was separated with a strong NdFeB permanent magnet, repeatedly washed with deionized water and finally solubilized in ultrapure water.

2.3. Characterization of the obtained nanostructures

2.3.1. XRD

X-ray diffraction analysis (XRD) was performed on a Shimadzu XRD 6000 diffractometer at room temperature. XRD is a rapid analytical technique primarily used for phase identification of a crystalline material and measurement of sample purity [24]. 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°.

2.3.2. TGA

The thermogravimetric (TG) analysis of the Fe_3O_4@ATB and Fe_3O_4 was assessed with a Shimadzu DTG-TA-50H instrument. TG analysis records the weight changes in a sample with respect to the temperature [25]. In this purpose, samples were screened to 200 mesh prior to analysis, were placed in alumina crucible, and heated with 10 K min^{-1} from room temperature to 800 °C, under the flow of 20 mL min^{-1} dried synthetic air (80% N_2 and 20% O_2).

2.3.3. FT-IR

A Nicolet 6700 FT-IR spectrometer (Thermo Nicolet, Madison, WI) connected to software of the OMNIC operating system (Version 8.0 Thermo Nicolet) was used to obtain FT-IR spectra of hybrid materials. The samples were placed in contact with attenuated total reflectance (ATR) on a multibounce plate of ZnSe crystal at controlled ambient temperature (25 °C). FT-IR spectra were collected in the frequency range of 4000–650 cm^{-1} by co-adding 32 scans and at a resolution of 4 cm^{-1} with strong apodization. The spectra were recorded as absorbance values at each data point in triplicate.

2.3.4. Particle size analysis

Particles size were determined by using dynamic light scattering technique (Zetasizer Nano ZS, Malvern Instruments Ltd., U.K.), at a scattering angle of 90° and 25 °C. 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.

2.4. Qualitative and quantitative assessment of the influence of Fe_3O_4@ATB on planktonic cells growth

The study of antimicrobial activity of Fe_3O_4@ATB against *E. faecalis* ATCC 29212 reference strain was performed by a qualitative method for antimicrobial susceptibility testing based on disk diffusion following CLSI 2012 recommendations. In brief, the inoculum represented by a bacterial suspension from a 16–18 h culture developed on solid medium, and adjusted according to McFarland 0.5 standard was seeded on a Muller-Hinton Agar (MHA) medium plate. After inoculation plates were left standing for 10 min to let the culture get absorbed. Afterwards, the antibiotic disks, and antibiotic disks plus 10 μL of Fe_3O_4@ATB, respectively were placed at corresponding distance on MHA plates, and plates were incubated la 35 ± 2 °C. The results reading were performed by comparatively measuring of inhibition zone diameter generated by different antibiotics, and antibiotic disks plus 10 μL of Fe_3O_4@ATB, respectively, according with their dimensions from CLSI, 2012. For these experiments there were used penicillin, vancomycin and streptomycin.

The quantitative method for the minimal inhibitory concentration (MIC) assay of each naonosystem (Fe_3O_4@ATB) consisted of two-fold microdilutions of nanoparticules stock solutions prepared in sterile saline were performed in liquid culture medium (nutrient broth) distributed in 96 multi-well plates. The concentration of

Table 1
ATB concentration in the initial solution of Fe_3O_4@ATB and ATB concentration from Fe_3O_4@ATB determined by TG analysis.

Fe_3O_4@ATB	The ATB concentration in the initial solution of Fe_3O_4@ATB (μg/mL)	ATB concentration (%) from Fe_3O_4@ATB determined by TGA at 600 °C
Penicillin (P)	4.5	1.9
Streptomycin (S)	7.8	2.4
Vancomycin (VA)	453	5.6

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