ANTI-BIOFILM ACTIVITY OF DEXTRAN COATED IRON OXIDE NANOPARTICLES

Alina Mihaela PRODAN[1], Ecaterina ANDRONESCU[2], Roxana TRUŞCĂ[3], Mircea BEURAN[4], Simona Liliana ICONARU[5*], Emil Ştefan BARNA[6], Mariana Carmen CHIFIRIUC[7], Luminița MARUTESCU[8]

Dextran coated iron oxide magnetic nanoparticles (DIOM-NPs) have been widely used in a variety of biomedical applications such as magnetic separation, magnetic resonance imaging, hyperthermia, magnetically-guided drug delivery, tissue repair, and molecular diagnostics. DIOM-NPs were synthesized by coprecipitation method. The aim of this study was to evaluate their anti-adherence activity on in vitro experimental biofilms formed by Pseudomonas aeruginosa and Enterococcus faecalis bacterial strains.

Keywords: antibiofilm activity, dextran, iron oxides, nanoparticles

1. Introduction

Nowadays, nanotechnology has developed to such extent that it has become possible to develop, characterize and control the properties of various particles even at a nanometric scale [1-4]. Recent studies state that inorganic nanometric particles have proved to be the building blocks in the area of nanoengineered particles. The most studied category of inorganic materials due to their unique optical, electrical, electronic and biological properties are the ones possessing magnetic behavior [5-10]. In the last years more investigations with several types of iron oxides have been carried out in the field of nanosized magnetic particles (mostly maghemite, γ-Fe_2O_3, or magnetite, Fe_3O_4). Iron oxide

[1] PhD student, *Carol Davila* University of Medicine and Pharmacy, Emergency Hospital *Floreasca*, Faculty of Applied Chemistry and Materials Science, University POLITEHNICA of Bucharest, Romania, e-mail: alina.prodan@yahoo.co.uk
[2] Prof., Faculty of Applied Chemistry and Materials Science, University POLITEHNICA of Bucharest, Romania, e-mail: ecaterina.andronescu@upb.ro
[3] PhD Eng, S.C. METAV R&D S.A., Bucharest, Romania, e-mail: truscaroxana@yahoo.com
[4] PhD student, *Carol Davila* University of Medicine and Pharmacy, Emergency Hospital *Floreasca*, Bucharest, Romania, e-mail: drmirceabeuran@yahoo.com
[5] Assistant Researcher, Faculty of Physics, University of Bucharest, National Institute of Materials Physics, Bucuresti-Magurele, Romania, e-mail: simonaiconaru@gmail.com
[6] Prof., Faculty of Applied Chemistry and Materials Science, University POLITEHNICA of Bucharest, Romania, e-mail: barna_emil@yahoo.com
[7] Asc. Prof., Faculty of Biology, Microbiology Department, University of Bucharest, Bucharest, Romania, e-mail: carmen_balotescu@yahoo.com
[8] Lect. PhD, Faculty of Biology, Microbiology Department, University of Bucharest, Bucharest, Romania, e-mail: lumidascalu@yahoo.com

nanoparticles have been of great interest, for their fundamental properties, their technological applications, such as high density magnetic recording media, sensors, catalysts, and clinical uses [11-14]. Because of the frequent tendency of magnetic nanoparticles to form aggregates, a special surface coating is required. To control the surface properties of iron oxide nanoparticles, coating is applied with a biocompatible polymer during or after the synthesis process [15]. Dextran ($C_6H_{10}O_5$), a branched polysaccharide, is commonly used to coat nanoparticles. Dextran coated iron oxide nanoparticles have been used for many purposes, including as MRI (Magnetic resonance imaging) contrast agents, to investigate nanoparticle accumulation and cellular uptake in malignant neoplasms *in vivo*, and to transform nanoparticles into active, targeted probes [13-14].

The emergence of antibiotic resistant bacterial strains and the severity of microbial infections associated with the biofilms forming on the prosthetic medical devices has led to new developments in the area of nanomaterials with antimicrobial activity [16-17].

The IO-NPs (iron oxide magnetic nanoparticles) were synthesized by an adapted co-precipitation method [18-20]. We have evaluated their antibacterial activity against biofilm embedded cells of the Gram-negative *Pseudomonas aeruginosa* and of the Gram-positive *Enterococcus faecalis* bacterial strains, by using culture based methods.

2. Methods and materials

2.1. Materials

Dextran, $H(C_6H_{10}O_5)_xOH$, (MW ~ 40,000) was purchased from Merck. Ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), ferric chloride hexahidrate ($FeCl_3 \cdot 6H_2O$), natrium hydroxide (NaOH), and hydrochloric acid (HCl) were purchased from Merck. De-ionized water was used in the synthesis of nanoparticles and for rinsing of clusters. The synthesis of iron oxide ferrofluid was carried out as reported in other papers [18-19].

2.2. Transmission electron microscopy

Transmission electron microscopy (TEM) images for the DIOM-Nps samples were recorded with a FEI Tecnai 12 equipped with a low dose digital camera from Gatan. The specimen for TEM imaging was prepared from the particle suspension in deionized water. A drop of well dispersed supernatant was placed on a carbon-coated 200-mesh copper grid, followed by drying the sample at ambient conditions before it was attached to the sample holder on the microscope.

2.3. Scanning electron microscopy

The morphology of the DIOM-NPs samples was investigated using a HITACHI S2600N-type scanning electron microscope (SEM), at 25 kV in vacuum. The elemental local analysis was performed using an energy dispersive

spectroscopy (EDS) detector from EDAX. Operating conditions were an accelerating voltage between 2 up 25 kEv (depending of the ratio signal/noise) with samples tilted at 25° to get the optimal take off angle (30°) allowing a dead time around 20–30% and a collecting time of 90–120 s.

2.4. *In vitro* qualitative screening of the antimicrobial activity

The *in vitro* qualitative screening of the antimicrobial activity was carried out by an adapted agar diffusion technique using a bacterial suspension of 0.5 McFarland density obtained from 24 hours cultures. The antimicrobial activity of the nanosized magnetic materials was determined against clinical and American Type Culture Collection (ATCC) reference microbial strains, i.e. *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis, Staphylococcus aureus* 0364, *Escherichia coli* ATCC 25922, *Escherichia coli* 714, *Enterobacter cloacae* 61R, *Pseudomonas aeruginosa* 1671, *Klebsiella pneumoniae* 2968 and *Candida krusei* 963. The microbial strains identification was confirmed with the aid of VITEK II automatic system. VITEK cards for identification and susceptibility testing (GNS-522) were inoculated and incubated according to the manufacturer's recommendations. The compounds were solubilized in dimethyl sulfoxide to a final concentration of 10 mg/mL. A volume of 10 μL of each tested compound solution was distributed directly on the solid medium previously seeded with the microbial inoculants. The inoculated plates were incubated for 24 hours at 37°C. Antimicrobial activity was assessed by measuring the growth inhibition zones diameters expressed in mm [22-23]. Following the results of the qualitative screening, only the microbial strains proving to be susceptible have been further tested in the anti-biofilm assay.

2.5. Anti-biofilm activity

The anti-biofilm activity of the tested compounds was tested by the microtiter method. For this purpose, the microbial strains have been grown in the presence of two-fold serial dilutions of the tested compounds performed in liquid nutrient broth/ YPG (yeast peptone glucose), distributed in 96-well plates and incubated for 24 hours at 37°C f. At the end of the incubation period, the plastic wells were emptied, washed three times with phosphate buffered saline (PBS), fixed with cold methanol and stained with 1% violet crystal solution for 30 minutes. The biofilm formed on plastic wells was resuspended in 30% acetic acid. The intensity of the colored suspensions was assessed by measuring the absorbance at 492 nm [23].

3. Results and discussions

Fig. 1 (A) shows the magnified TEM image of dextran coated iron oxide nanoparticles (DIOM-NPs) obtained by coprecipitation. The image is clearly

showing that the samples have an uniform morphology with relatively spherical shape at nanometric size. The high-resolution TEM image in Fig. 1 (B) shows the internal crystalinity for an iron oxide nanocrystal. The clear lattice fringe in the High-resolution transmission electron microscopy (HRTEM) image demonstrates the well crystalline nature of the samples. Regular fringes can be observed with a spacing of 0.251 nm, coresponding to the (311) interplanar distance of the cubic maghemite.

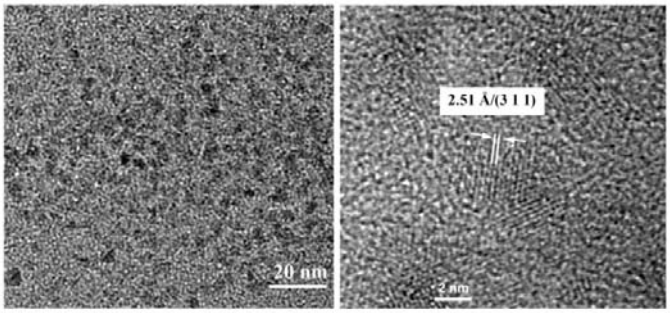

Fig. 1. Magnified TEM image showing a homogeneous distribution of dextran coated iron oxide nanoparticles (A), HRTEM micrographs (B)

Fig. 2 (top) presents the scanning electron microscopy image for dextran coated iron oxide nanoparticles.

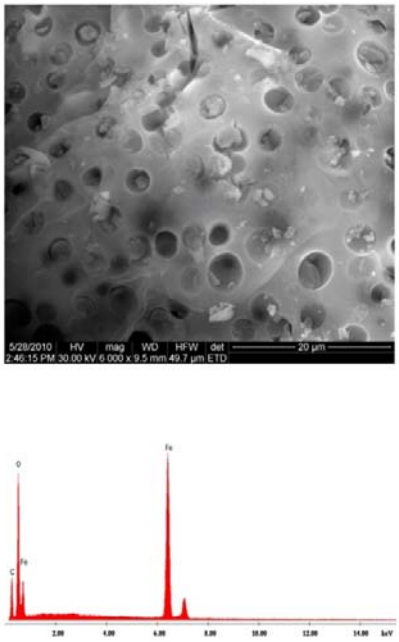

Fig. 2. Scanning electron microscopy image of DIOM-NPs (top), EDAX spectra of DIOM-NPs (bottom)

The SEM image revealed an uniform distribution of the samples. The EDAX spectrum shown in Fig. 2 (bottom) confirms the presence of carbon (C), oxygen (O) and iron (Fe) in the investigated DIOM-NPs samples.

The qualitative *in vitro* screening of the antimicrobial activity of the DIOM-NPs compounds performed with stock solutions of 10 mg/mL obtained in dimethylsulfoxide (DMSO) allowed the selection of the active compounds.

The occurence of the growth inhibition zones around the spotted DIOM-NPs with larger diameter than for those of the solvent was recorded as a positive result (Fig. 3).

Fig. 3. Qualitative assay of the inhibitory activity of DIOM-NPs on E. *faecalis* and P. a*eruginosa 1397* growth.

The results of the *in vitro* screening of the antimicrobial activity against a large spectrum of bacterial and fungal strains revealed that the obtained nanoparticles clearly inhibited the growth of *Pseudomonas aeruginosa* and *Enterococcus faecalis* strains (**Table 1**), which have been further tested for the anti-biofilm activity.

Table 1

***In vitro* qualitative screening of the antimicrobial activity of DIOM-NPs**

Tested strains	DIOM-NPs
P. aeruginosa 1397	+
E.coli 714	-
E.coli ATCC 25922	+-
K. pneumoniae 2968	-
B. subtilis	-
E. cloacae 61R	-
E. faecalis ATCC 29212	+
S. aureus 0364	-
C. krusei 963	+-

The tested nanoparticles exhibited a remarkable anti-biofilm effect manifested till very low concentrations, i.e. 0.01mg/ml (Fig. 4). This inhibitory effect was noticed for both P. *aeruginosa* and *E. faecalis* biofilms, as revealed by

the assessment of the biofilm embedded cells, in comparison with the positive control represented by the microbial biofilm developed in the absence of the tested nanoparticles.

Nowadays antibiotic resistance has become a serious public health problem. As a result of the rapid resistance development to the existing portfolio of antimicrobial drugs, there is an increasing need to design new antibacterial and antifungal agents with better activity profiles and lower toxicity [21].

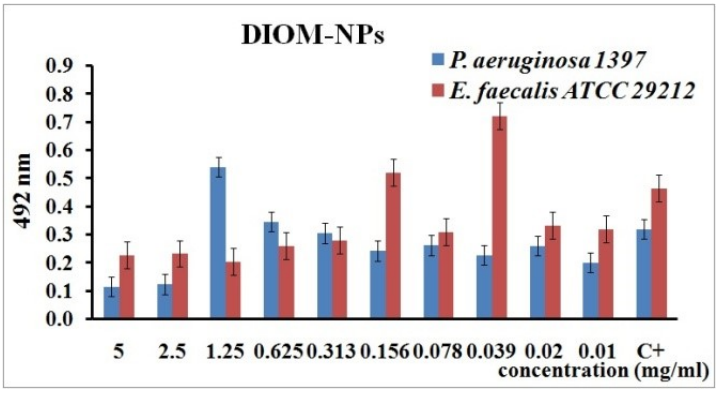

Fig. 4. Quantitative assay of the inhibitory effect of dextran oxide on biofilms developed on the inert substratum by *P. aeruginosa* and *E. faecalis* strains, quantified by the A_{492nm} values (C+ = positive control).

Bionanotechnology investigates the interactions between nanoscale materials and biological systems and creates the technologies for interfacing the two. Inorganic metal oxides may serve as effective disinfectants, due to their relatively non-toxic profile, chemical stability and efficient antibacterial activity [24].The insertion of the prosthetic medical devices for different exploratory or therapeutically purposes, especially in severe pathological conditions, represents a risk factor for the occurrence of chronic infections in developed countries, being characterized by slow onset, middle intensity symptoms, chronic evolution and resistance to antibiotic treatment [25]. The microbial species of clinical interest, often involved in biofilm associated diseases are belonging to a very large spectrum, from the *Gram positive* (*S. epidermidis* and *S. aureus*) to the *Gram negative* pathogens (*P. aeruginosa, E.coli*) and to different members of the *Candida* genus [26]. The understanding of microbial infections related to the biofilm development on tissues or indwelling devices was possible by using different qualitative and quantitative *in vitro* specific assays. In our study we have also used both assays for studying the susceptibility of microbial cells grown in suspension, called planktonic as well as of those developed in biofilms, called sessile cells.

The qualitative screening of the tested compounds at 10 mg/ml concentration presented in Table 1 showed that they were slightly active only on two of the tested strains, i.e. *E. faecalis 29212* and *P. aeruginosa 1397.* In order to investigate the dose-concentration effects of the tested oxides, we have further chosen these strains to be tested in the presence of a range of two-fold serial dilutions of the tested compounds. The stimulatory effect of DIOM-NPs, especially noticed in the presence of high concentrations, on the microbial growth rate of the tested strains is probably due to the potential of these strains to use the iron oxide as a source of iron ions required for the microbial metabolism and pathogenicity. Studies in the last decades on microbial adherence to different substrata led to the conclusion that the survival of microorganisms in the natural habitats, including medical ecosystems, strongly depends on their capacity to adhere to different surfaces/substrata and to form biofilms. A biofilm is a sessile microbial community composed of cells embedded in a matrix of extracellular polymeric substances attached to a substratum or interface. The matrix is primarily of microbial origin and the cells encased in this matrix present a modified phenotype, being metabolically more efficient and well protected, exhibiting resistance to different stress factors, including host defense mechanisms and antibiotics in agreement with previous studies [27]. Concerning the antimicrobial activity on cell growth in biofilms, quantified by measuring the absorbance of the adherent cells colored suspension at 492 nm, the DIOM-NPs presented an inhibitory effect on the ability of the *Gram-negative P. aeruginosa* and the *Gram-positive E. faecalis* strains to develop biofilms. The effect of DIOM-NPs on the planktonic microbial growth observed by the absorbance measurements at 620 nm was reported in Iconaru et al, Nanoscale Res Lett [28].

The specific antimicrobial activity revealed by the qualitative assay demonstrates that our compounds are interacting differently with the microbial targets, probably due to the differences in the microbial wall structures. The first step in the microbial cell-nanoparticle interaction is to attach and anchor to the surface of the cell wall, involving both electrostatic forces and molecular interactions. The *Gram-negative* bacteria possess an outer membrane with porins and a unique periplasmic space not found in *Gram-positive* bacteria, favoring the internalization of nanoparticles and their intracellular accumulation. The tested metal nanoparticles seemed to exhibit a better bactericidal effect on the *Gram-negative* strains, as compared with the *Gram-positive* ones, accounting for the hypothesis that they cause changings in bacterial membrane permeability, affecting the physiology and finally, the cell viability [27].

4. Conclusions

The Dextran coated iron oxide magnetic nanoparticles (DIOM-NPs) prepared using a simple adapted coprecipitation chemical method have a relatively spherical shape and nanometric size considerably smaller than that reported in the literature.The transmision electron microscopy results revealed a uniform distribution of the nanoparticles and the crystalline form of the cubic maghemite. The elemental composition of the samples was emphasized by EDAX studies. The antimicrobial results showed that the tested DIOM-NPs interact differently with the microbial cells in different growth states, i.e. suspension versus biofilm embedded cells, demonstrating the specificity of the molecular interactions established between the microbial cells and the tested nanoparticles. The anti-biofilm activity was superior to that antimicrobial one in the case of these nanoparticles, so they could be successfully used for further development of novel antimicrobial materials or strategies for fighting medical biofilms.

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