BACTERIAL ADHERENCE TO THE CELLULAR AND INERT SUBSTRATE IN THE PRESENCE OF $CoFe_2O_4$ AND Fe_3O_4 /OLEIC ACID – CORE/SHELL

C. CHIFIRIUC[a], V. LAZĂR[a], C. BLEOTU[a,b], I. CĂLUGĂRESCU[a],
A. M. GRUMEZESCU[b*], D. E. MIHAIESCU[a], D. E. MOGOŞANU[a],
A. S. BUTEICĂ[c], E. BUTEICĂ[c]
[a]*Faculty of Biology, University of Bucharest, Microbiology Immunology Department, Aleea Portocalilor 1-3, Sector 5, 77206-Bucharest, Romania*
[b]*Faculty of Applied Chemistry and Materials Science, "Politehnica" University of Bucharest, 1–7 Polizu Street, 011061 Bucharest, Romania*
[c]*Faculty of Pharmacy, University of Medicine and Pharmacy of Craiova, 66, 1 May Avenue, 200638 Craiova, Romania*
[d]*Stefan S Nicolau Institute of Virology, 285 Mihai Bravu Avenue, 030304, Bucharest*

The purpose of the present work is the *in vitro* assay of the efficiency of magnetic nanoparticles of $CoFe_2O_4$ and Fe_3O_4/oleic acid – core/shell against bacterial adherence to cellular and acellular substrata and against bacterial biofilm formation. Magnetic nanoparticles have been synthesized by Massart method with oleic acid as the surfactant, under domestic microwave conditions. The dimensions of Fe_3O_4 and $CoFe_2O_4$ nanoparticles were in the 5-20 nm range and they were characterized by High Resolution Transmision Electron Microscopy. The adherence assays were performed on Gram negative (*Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa*) and Gram-positive (*Staphylococus aureus, Entereococcus* faecalis) bacterial strains as well as on yeasts (*Candida albicans*). Our results showed that the magnetic nanoparticles of $CoFe_2O_4$ and Fe_3O_4 / oleic acid – core/shell exhibit specific influences on the ability of different microbial strains to colonize the cellular and inert substrata. They either stimulate or inhibit this hallmark of microbial virulence, depending on the tested nanoparticles composition and the tested strain. It is to be mentioned that both types of nanoparticles have produce a strong inhibitory effect on the adherence to the cellular substrate of *S. aureus* and *C. albicans* strains, pathogens frequently implicated in the human pathology and in the etiology of biofilm associated infections. These results are leading us to the hypothesis that magnetic nanoparticles could be used for the development of novel antimicrobial materials or strategies for fighting medical biofilms.

(Received November 28, 2010; accepted December 7, 2010)

Keywords: Magnetic nanoparticles, Core/shell, Cellular substrat, HeLa cells, Bacterial adherence

1. Introduction

Carbon nanotubes are considered potential biomedical materials because of their flexible structure and their tendency for functionalization. Bio-nanotechnology investigates the interactions between nanoscale materials and biological systems and creates the technologies for interfacing the two. Potential products of bio-nanotechnology in the pharmaceutical industry include nano-medicines and their components such as: additives, delivery devices [1], carriers [2], etc. The antibacterial property of metallic nanoparticles has been a much debated research subject. *Escherichia coli, Salmonella typhimurium, Bacillus subtilis* and *Pseudomonas aeruginosa* [3], [4]

*Corresponding author: grumezescu@yahoo.com

are a few examples of pathogenic agents on which the antibacterial activity of nanoparticles was studied. On a more particular note, Singh et al. [5] reported silver nanoparticles to have pronounced effects against Gram-negative and Gram-positive organisms. The bacteriostatic activity and the cytotoxicity of (non)magnetic nanoparticles based on C-Fe, C-Cu, C-Al and C-C was also studied by Buteica et al. [6] on two reference strains of *Staphylococcus aureus* and *E. coli*.

The last decades studies of microbial adherence to different substrata were leading to the conclusion that the survival of microorganisms in the natural habitats, including medical ecosystems, is dependent 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 defence mechanisms and antibiotics [7]. The substratum of adherence can be cellular or acellular, including all types of biomaterials used in medicine, microbial adhesion representing an early step of infectious diseases pathogenesis, which is a precondition of tissue and medical devices colonization and biofilm's formation [8-11].

The interference with the microbial adherence to different substrata is considered an efficient way for controlling the severity of biofilm associated infections, which are now considered at the average level of 65 % of total number of infections.

Recent research has proposed the use of nanoparticles for the development of different biomaterials with antibacterial properties [12]. The purpose of the present work is the *in vitro* assay of the efficiency of magnetic nanoparticles of $CoFe_2O_4$ and Fe_3O_4/ oleic acid – core/shell against bacterial adherence to cellular and acellular substrata and against bacterial biofilm formation.

2. Materials and methods

2.1 Preparation and characterization of Fe_3O_4 and $CoFe_2O_4$ / oleic acid core/shell

Magnetic nanoparticles have been synthesized through the Massart method using Fe^{3+} and Fe^{2+} salts with oleic acid as the surfactant, under microwave conditions. Transmission Electron Microscope (TEM) confirmed the formation of magnetic naoparticles/oleic acid - core/shell in the range of 5-20 nm [13].

The *in vitro* biological screening effects were tested against bacterial strains, both Gram negative (*E. coli, Klebsiella pneumoniae, P. aeruginosa)* and Gram-positive (*S. aureus, Enterococcus faecalis)* as well as fungal (*Candida albicans)* reference and clinical strains.

2.2 Study of the adherence to the cellular substrate represented by HeLa cells (Cravioto's adapted method)

In this purpose, HeLa cells were routinely grown in Eagle's minimal essential medium (Eagle MEM) supplemented with 10% heat-inactivated (30 min at 56°C) foetal bovine serum (Gibco BRL), 0.1 mM nonessential amino acids (Gibco BRL), and 0.5 ml of gentamycin (50 µg/ml) (Gibco BRL) and incubated in a 5% CO_2 humid atmosphere, at 37°C for 24 hrs [14]. HeLa cell monolayers grown in 6 multi-well plastic plates were used at 80-100% confluence. Bacterial strains from an overnight culture on 2% agar nutrient were suspended in Eagle MEM with no antibiotics and adjusted to a final density of 10^7 CFU/ml. The HeLa cell monolayers were washed 3 times with Phosphate Buffered Saline (PBS) and 2 ml from the bacterial suspension were inoculated in each well. The inoculated plates were incubated for 3 hrs at 37ºC. After incubation, the monolayers were washed 3 times with PBS, briefly fixed in cold ethanol (3 minutes), stained with Giemsa stain solution (1:20) (Merck, Darmstadt, Germany) and incubated for 30 min. The plates were washed, dried at room temperature overnight, examined microscopically (magnification, ×2500) with the immersion objective (IO) and photographed with a Contax camera adapted for Zeiss (Axiolab 459306) microscope [15,16].

Three distinct patterns of adherence have been investigated during this study: localized adherence (LA), in which the bacteria attach to and form microcolonies in distinct regions of the surface; diffuse adherence (DA), in which bacteria adhere evenly to the whole cell surface, and aggregative adherence (AggA), in which aggregated bacteria attach to the cell in a stacked-brick arrangement [19]. The adherence index was expressed as the ratio between the number of the eukaryotic cells with adhered bacteria: 100 eukaryotic cells counted on the microscopic field.

2.3 Study of adherence to the inert substrate

96-multi well plastic plates containing binary dilutions of the tested compounds in a final volume of 200 µl were inoculated with 50 µl microbial suspensions of 10^7 CFU/ml prepared in sterile saline and inoculated and incubated for 24 h at 37^0C. After incubation, the wells were discarded, washed three times by PBS and the bacterial cells adhered to the plastic walls were stained by 1% violet crystal solution for 15 min. The coloured biofilm was thereafter fixed by cold methanol for 5 minutes and resuspended by 33% acetic acid solution. The A_{490} of the blue suspension was measured using an ELISA reader, the obtained values being proportional with the number of the adhered bacterial cells [14, 17].

3. Results and discussion

3.1 Influence of Fe_3O_4/oleic-acid core/shell on bacterial biofilms development

Bacterial strains implicated in human infectious pathology are using a large arsenal of different virulence factors to subvert the host cellular functions, but all of them possess the ability to adhere to the epithelial cells in order to initiate the infectious process, to colonize the host and to overcome the natural host defence mechanisms (fluid secretions, blood flow, intestinal peristaltism, coughing, sneezeing etc.) [18]. Microorganisms involved in biofilm associated human infections are ranging from Gram positive pathogens, such as *Staphylococcus sp.* and *Enterococcus sp.* to Gram negatives, including *P. aeruginosa, E. coli* amongst others and fungal strains, particularly *C. albicans*. The microbial biofilms can develop on tissues teguments and mucosa, intact or damaged, teeth, bones, generating a large spectrum of chronic, hard to treat infections. Using a simple experimental model represented by HEp-2 cells [15, 19] we have studied the influence of magnetic nanoparticles of $CoFe_2O_4$ and Fe_3O_4) / oleic acid – core/shell on the ability of the above mentioned human pathogenic strains to adhere to the respective epithelial cell line.

We have observed that the tested strains exhibited a similar behaviour in relation with the two types of tested nanoparticles (Fig. 1). A significant inhibitory effect on the adherence to the cellular substrate was observed in the case of *S. aureus* and *Candida albicans.* Althoug the intensity of adherence process was not significantly influenced in the case of *E. coli, P. aeruginosa* and *E. faecalis,* however, a slight stimulation was observed in the case of *P. aeruginosa* in the presence of both nanoparticle types and a slight inhibition of adherence in the case of *E. coli* strain induced by $CoFe_2O_4$. These results demonstrate the specificity of the molecular interactions established between the microbial wall's components and the tested nanoparticles. The Fe_3O_4 nanoparticles induced changes in the adherence pattern of the tested bacterial strains, i.e. the occurrence of the localized pattern in *E. coli* and *S. aureus* and of the aggregative pattern in the case of *P. aeruginosa* strain. Microbial biofilms can also develop on acellular substrata, including the surface of medical devices (therapeutical or exploratory). *In vitro* adhesion of microorganisms to biomaterial surfaces has been extensively studied and it has been shown to require both non-specific reversible interactions and highly specific irreversible interactions. Initial attachment, or reversible adhesion, of microorganisms to a biomaterial surface is dependent upon the physical characteristics of the microorganisms, the biomaterial and the surrounding environment. The irreversible adhesion of the microorganisms to biomaterials occurs with the binding of specific microbial adhesins to receptors expressed by the conditioning film. In the experimental model used for testing the microbial strains for their adherence capacity and biofilm developing potential

on plastic wells wall, the biofilms formed on the wall was fixed and stained, the color intensity being quantified by measuring the absorbance at 492 nm, the obtained value being proportional with the intensity of bacterial growth in *adherent phase*. Significant results were obtained for the Gram-positive tested strains, i.e. *S. aureus* and *E. faecalis*, which exhibited a better adherence to the plastic substrate in the presence of $CoFe_2O_4$, while the ability of *C. albicans* to colonize the inert substratum was enhanced in the presence of Fe_3O_4 nanoparticles (Fig. 1).

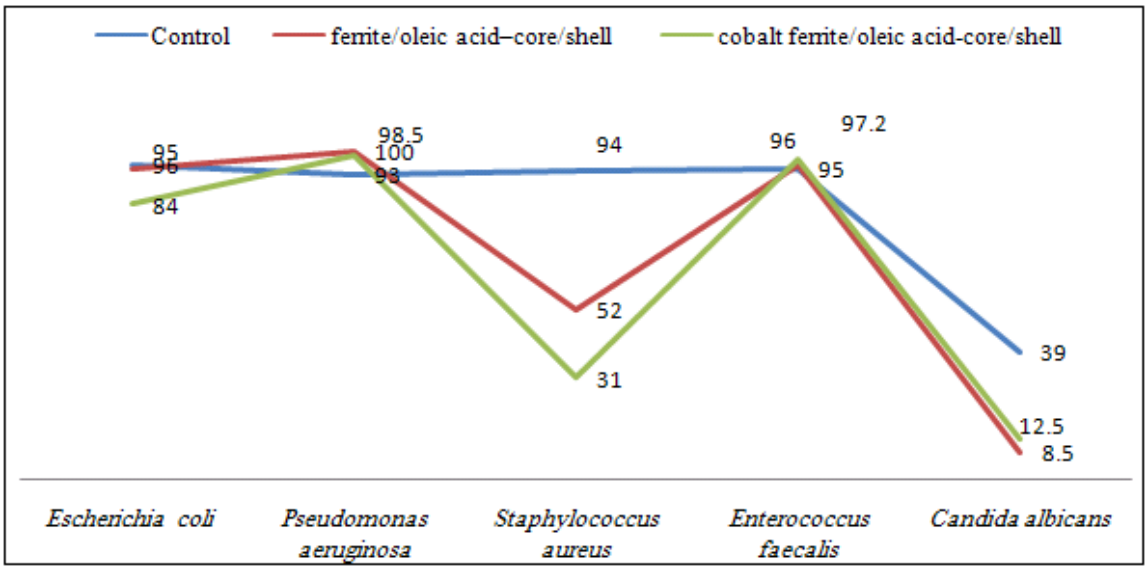

Fig. 1 Adherence indexes obtained for the microbial tested strains in the presence of magnetic nanoparticles

Table 1: The adherence pattern exhibited by the tested microbial strains in the presence of the tested nanoparticles

Microbial strain		Control	Fe_3O_4 / oleic acid – core/shell	$CoFe_2O_4$ / oleic acid – core/shell
Gram – negative bacteria	*Escherichia coli*	DA AggA	***LA**-AggA DA	DA AggA
	Pseudomonas aeruginosa	DA LA	DA LA-**AggA**	DA
		DA LA	DA LA-**AggA**	DA
Gram-positive bacteria	*Staphylococcus aureus*	DA-AggA	DA **LA**-AggA	DA AggA
	Enterococcus faecalis	LA AggA	LA AggA	LA AggA
Fungal strains	*Candida albicans*	LA	LA	LA

* in red- the changes occurred in adherence patterns in the presence of nanoparticles.

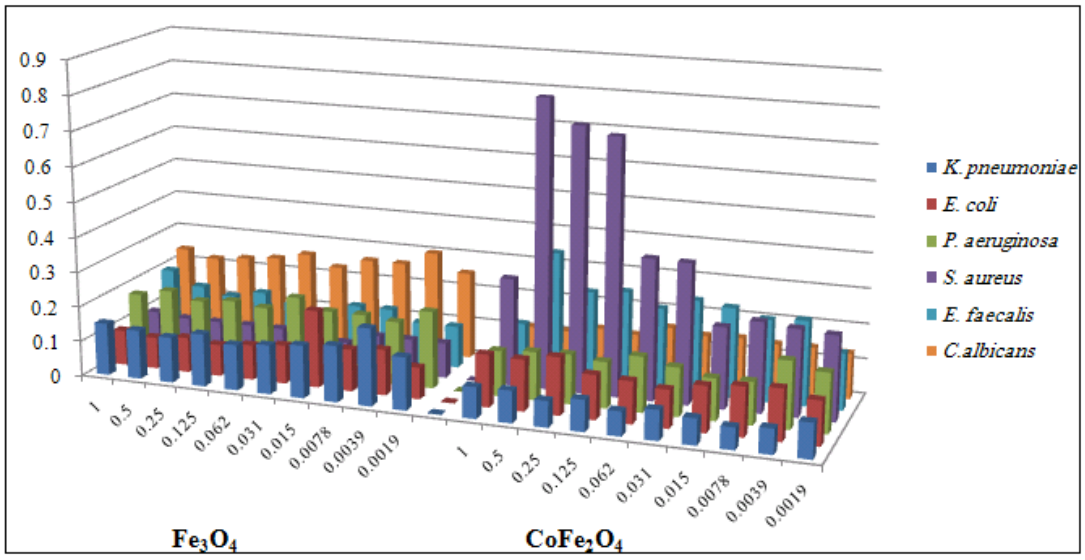

Fig. 2. Influence of Fe_3O_4 and $CoFe_2O_4$ on the microbial strains ability to develop biofilms on plastic substrata

4. Conclusion

Our results showed that the magnetic nanoparticles of $CoFe_2O_4$ and Fe_3O_4 / oleic acid – core/shell exhibit specific influences on the ability of different microbial strains to colonize the cellular and inert substrata, either by stimulating or inhibiting this hallmark of microbial virulence, depending on the tested nanoparticles composition and the tested strain. It is to be mentioned that both types of nanoparticles have a strong inhibitory effect on the adherence to the cellular substrate of *S. aureus* and *C. albicans* strains, pathogens frequently implicated in the human pathology and in the etiology of biofilm associated infections. These results are leading us to the hypothesis that magnetic nanoparticles could be used for the development of novel antimicrobial materials or strategies for fighting medical biofilms.

References

[1] S. Shrivastava, Digest Journal of Nanomaterials and Biostructures **3**, 257 (2008)
[2] M. Veerapandian, K.Yun, Digest Journal of Nanomaterials and Biostructures, **4**, 243 (2009)
[3] R.M.Tripathi, A. Saxena, N. Gupta, H. Kapoor, R. P. Singh, Digest Journal of Nanomaterials and Biostructures, **5**, 323 (2010)
[4] K. Natarajan, S. Selvaraj, V. Ranachandra, Digest Journal of Nanomaterials and Biostructures **5**, 135 (2010)
[5] M. Singh, S. Singh, S. Prasad, I. S.Gambhir, Digest Journal of Nanomaterials and Biostructures, **3**, 115 (2008)
[6] A. S. Buteică, D. E. Mihaiescu, A. M. Grumezescu, B. Ş. Vasile, A. Popescu, D. Călina, O. M. Mihaiescu, Digest Journal of Nanomaterials and Biostructures, **5**, 651 (2010)
[7] RM Donlan, JW Costerton, Clinical Microbiol. Rev. **15**, 167 (2002)
[8] C Cucarella, C Solano, J Valle, B Amorena, Í Lasa, JR Penadés, J. Bacteriol. **183**, 2888 (2001)
[9] DR Cundell, E. Tuomanen, Virulence mechanisms of bacterial pathogens, Sec. Ed., Washington, D.C., Asm Press, (1995)
[10] V. Livrelli, C. De Champs, P. Di Martino, A. Darfeuille-Michaud, C. Forestier, B. Joly, J. Clin. Microbiol., **34**, 1963 (1996)
[11] J. Yatsuyanagi, S. Saito, K. Sato, Y. Miyajima, K.I. Amano, K. Enomoto, J. Clin. Microbiol., **40**, 294 (2002)
[12] E Weir , A Lawlor , A Whelan , F Regan, Analyst.**133**, 835, (2008)

[13] A. S. Buteică, D. E. Mihaiescu, A. M. Grumezescu, B. Ş. Vasile, A. Popescu, O. M. Mihaiescu, R. Cristescu, Digest Journal of Nanomaterials and Biostructures, **5**, 975 (2010)
[14] C Chifiriuc, V Lazar, O Dracea, LM Ditu, D Smarandache, M Bucur, C Larion, R Cernat, E Sasarman, Int. J. Mol. Sci. **8**, 583 (2007.)
[15] V. Lazar, M.C. Balotescu, D. Smarandache, T. Vassu, E. Sasarman, L.M. Petrache, M. Orasanu, R. Cernat, Roum. Biotech. Lett. **9**, 1675 (2004)
[16] V. Lazar, M.C. Balotescu, D. Smarandache, T. Vassu, E. Sasarman, L.M. Petrache, M. Orasanu, R. Cernat, Roum. Biotech. Lett. **9,** 1669 (2004)
[17] Chifiriuc MC, Bleotu C, Ditu LM, Banu O, Bleotu C, Drãcea O, Bucur M, Larion C, Israil AMI, Lazãr V., Roum. Arch. Microbiol. Immunol. **68**, 28 (2009)
[18] M. Croxen , BB. Finlay, Nature Reviews Microbiology (2009)
[19] A. Cravioto, R. J. Gross, S.M. Scotland, B. Rowe, Curr. Microbiol. **3**, 95 (1979)