

# Local delivery of tetracycline from $\gamma$ -aminobutyric acid-silica networks thin films for preventing microbial colonization

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Alina Maria Holban<sup>1,2</sup>, Ecaterina Andronescu<sup>2</sup>, Valentina Grumezescu<sup>2,3</sup>, Gabriel Socol<sup>3</sup>, Alexandru Mihai Grumezescu<sup>1,\*</sup>, Veronica Lazăr<sup>2</sup>, Mariana Carmen Chifiriuc<sup>2</sup>

<sup>1</sup> Department of Science and Engineering of Oxide Materials and Nanomaterials, Faculty of Applied Chemistry and Materials Science, University Politehnica of Bucharest, 1-7 Polizu Street, 011061 Bucharest, Romania

<sup>2</sup> Microbiology Immunology Department, Faculty of Biology, University of Bucharest, 1-3 Portocalilor Lane, Sector 5, 77206 Bucharest, Romania

<sup>3</sup> Lasers Department, National Institute for Lasers, Plasma & Radiation Physics, P.O. Box MG-36, Magurele, Bucharest, Romania

\*corresponding author e-mail address: [grumezescu@yahoo.com](mailto:grumezescu@yahoo.com)

## ABSTRACT

The purpose of this study was to characterize and evaluate the cytotoxicity and antimicrobial activity of  $\gamma$ -aminobutyric acid-silica/tetracycline network thin films prepared by Matrix Assisted Pulsed Laser Evaporation (MAPLE). Thin films were characterized by Infrared Microscopy (IRM), X-ray Diffraction (XRD), Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM). TEM images showed an average size of gamma-aminobutyric acid-silica network ( $\gamma$ -AA/SiO<sub>2</sub>) lower than 10 nm. Unlike the dropcast samples, IRM recorded on MAPLE films revealed a good distribution of monitored functional groups on the entire scanned surface. Besides a good homogeneity of the coatings, SEM analysis revealed aggregates typical for MAPLE deposition, randomly distributed on the surface with an average diameter of ~300 nm. The biological evaluation of MAPLE nanobiocoated surfaces evidenced a good biocompatibility and an enhanced antibiofilm effect against both Gram positive (*Staphylococcus aureus*) and Gram negative (*Escherichia coli*) tested strains. Our results recommend the use of  $\gamma$ -aminobutyric acid-silica networks as matrixes for controlled local delivery of antibiotics, with practical applications in developing improved medical surfaces for the prevention or reduction of surface-associated microbial infections.

**Keywords:** antimicrobial surfaces, MAPLE, *S. aureus*, *E. coli*,  $\gamma$ -aminobutyric acid-silica networks.

## INTRODUCTION

Hospital surfaces contamination has a major impact in the transmission of healthcare-associated pathogens, especially multidrug resistant bacteria. Even though major interventions have been made in order to improve cleaning and disinfections in healthcare facilities, hospital-acquired infections remain a major cause of patient morbidity and mortality worldwide (Boyce, 2007; Klevens *et al.*, 2007). In the US only, it is estimated a number of 1.7 million healthcare-associated infections, which result in approximately 99 000 deaths per year (Klevens *et al.*, 2007). Studies demonstrated that main sources of pathogens causing hospital-acquired infections are: i) the patients' endogenous flora (40–60%); ii)

cross-infection via the hands of personnel and contaminated medical objects (20–40%); iii) antibiotic-driven modifications in patients' microbiota (20–25%); and iv) other, including contamination of the healthcare unit environment (20%) (Weber *et al.*, 2013). Studies proved that the contaminated surfaces in hospitals play an important role in the transmission of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus spp.* (VRE), *Clostridium difficile*, *Acinetobacter spp.*, and norovirus (Weber *et al.*, 2013).

The key aspects demonstrating the importance of surface contamination in the transmission of hospital - acquired infections are

explained by the following: i) surfaces in a rooms of colonized or infected patients are frequently contaminated with the pathogen; ii) the pathogen is able to survive long periods of time on hospital room surfaces and medical equipment; iii) the contact of healthcare personnel with infected medical surfaces or equipment leads to contamination of hands or gloves; iv) person-to-person transmission and shared medical equipment is the major cause of clonal outbreaks of pathogens contaminating the medical surfaces of colonized or infected patients (Weber *et al.*, 2013).

Furthermore, another risk condition for acquiring healthcare-associated infections is represented by patients with implanted medical and prosthetic devices. Due to their wide spread within the body and skin, Staphylococci and *Escherichia coli* account for the majority of device-associated infections (von Eiff *et al.*, 2005). Their ability to adhere to materials and to promote biofilms formation is the most important feature of their pathogenicity. Since biofilms are highly resistant structures to most antimicrobials and host defense system (Lazar, 2011), the removal of infected implanted devices is often inevitable.

To reduce the incidence of device-associated infections, specific guidelines including both technological and non technological strategies for prevention have been established (Cotar *et al.*, 2013; Grumezescu and Chifiriuc, 2014; Holban *et al.*, 2014). The surface modification of the implantable device may lead to a change in its specific and nonspecific interactions with microorganisms, and thus to the reduction of microbial adherence and biofilms formation. A new and promising strategy involves the impregnation of the devices with various natural and synthetic antimicrobial substances in order to prevent colonization (von Eiff *et al.*, 2005).

Matrix Assisted Pulsed Laser Evaporation (MAPLE) has proved a great impact on surface

coating, allowing the deposition of thin, uniform films, made of different nanostructured materials, polymers and active compounds (Grumezescu *et al.*, 2014c; Grumezescu *et al.*, 2014d).

$\gamma$ -aminobutyric acid ( $\gamma$ -AA) is a non-protein amino acid widespread in the environment. Being a bifunctional molecule,  $\gamma$ -AA can be used in nanotechnology to obtain nanostructured networks with low diameter pores (Grumezescu *et al.*, 2014a). Also, many bacteria, such as *Pseudomonas sp.*, lactic bacteria and *Escherichia coli*, can synthesize and release  $\gamma$ -AA, suggesting that this compound may act as a communication molecule between different, or between bacteria and their host (Dagorn *et al.*, 2013). Therefore, utilizing  $\gamma$ -AA for obtaining nanostructured materials with antimicrobial effect we can increase not only the quality of the material, by controlling the pore size, but also the efficiency of the material on bacteria, which are familiar with such compounds.

Silica networks as drug delivery system, were involved in the development of new formulations that control rate and period of drug delivery (Heikkila *et al.*, 2007; Li *et al.*, 2014; Peng *et al.*, 2013). Few studies reported the potential of silica networks to improve the antimicrobial effect of currently used antibiotics (Balaure *et al.*, 2013; Grumezescu *et al.*, 2013; Guzun *et al.*, 2014; Voicu *et al.*, 2013) and fewer studies report the laser processing of silica network in order to create antimicrobial surfaces (Mihaiescu *et al.*, 2013).

The purpose of this study was the fabrication, characterization and bioevaluation of  $\gamma$ -aminobutyric acid-silica network thin film prepared by MAPLE as a matrix for controlled local delivery of the antibiotic tetracycline, with practical applications in developing improved medical surfaces for the prevention or reduction of surface-associated infections.

## MATERIALS AND METHODS

### Materials and methods

The  $\gamma$ -aminobutyric acid (>99%), sodium metasilicate, sulfuric acid (ACS reagent 95–98%),

were purchased from Sigma–Aldrich, and were used without any further purification.

### Preparation of $\gamma$ -aminobutyric acid-silica network

The synthesis of  $\gamma$ -aminobutyric acid-silica ( $\gamma$ -AA/SiO<sub>2</sub>) network was performed using our previously reported protocol (Grumezescu *et al.*, 2014a).

### MAPLE target preparation and deposition of $\gamma$ -aminobutyric acid-silica networks@tetracycline thin films

400 mg  $\gamma$ -aminobutyric acid-silica networks and 100 mg tetracycline were dispersed in 30 mL DMSO then the solution was poured into a pre-cooled target holder and subsequently immersed in liquid nitrogen for 30 min. MAPLE depositions were carried out in a stainless steel chamber using a KrF\* laser source ( $\lambda = 248$  nm and  $\tau_{FWHM} = 25$  ns), model COMPexPro 205 (Lambda Physics-Coherent), that operated at a repetition rate of 15 Hz. A laser beam homogenizer was used to improve the energy distribution of the laser spot. The laser fluence was set at 300–500 mJ/cm<sup>2</sup> range whereas the laser spot area was set to 36 mm<sup>2</sup>. During the laser irradiation, the frozen target was rotated at a rate of 0.4 Hz to avoid the target heating and drilling. All depositions were conducted at room temperature into a background pressure of 1 Pa. The films were grown at a target-substrate separation distance of 5 cm by applying 140,000 subsequent laser pulses. During the deposition process, the target was kept at a temperature of ~173 K by active liquid nitrogen cooling. The coatings were deposited onto double side polished (100) silicon and glass substrates for physico-chemical analyses and biological assays, respectively. Prior to introduction inside the deposition chamber, the substrates were successively cleaned into an ultrasonic bath with acetone, ethanol and deionized water for 15 min. During the deposition, the substrates were continuously rotated. Thus, the  $\gamma$ -aminobutyric acid-silica networks/tetracycline was uniformly spread over the surface of the substrates. For data comparison, a control set of films were prepared by pouring of few drops onto the surface of double side polished (100) silicon.

### Characterization

#### XRD

X-ray diffraction analysis was performed on a Shimadzu XRD 6000 diffractometer at room temperature. In all the cases, K radiation from a Cu X-ray tube (run at 15 mA and 30 kV) was used. The samples were scanned in the (2 $\theta$ ) Bragg-Brentano geometry in the 10–40° range.

#### TEM

The transmission electron microscopy (TEM) images were obtained on finely powdered samples using a Tecna<sup>TM</sup> G2 F30 S-TWIN high resolution transmission electron microscope from FEI Company (OR, USA) equipped with SAED. The microscope operated in transmission mode at 300 kV with TEM point resolution of 2 Å and line resolution of 1 Å. The prepared powder was dispersed into pure ethanol and ultrasonicated for 15 min. After that, diluted sample was poured onto a holey carbon-coated copper grid and left to dry before TEM analysis.

#### IRM

IR mapping were recorded on a Nicolet iN10 MX FT-IR Microscope with MCT liquid nitrogen cooled detector in the measurement range 4000–700 cm<sup>-1</sup>. Spectral collection was made in reflection mode at 4 cm<sup>-1</sup> resolution. For each spectrum, 32 scans were co-added and converted to absorbance using OmnicPicta software (Thermo Scientific). Approximately 250 spectra were analyzed for each sample. One absorption peak known as being characteristics for the prepared material was selected as spectral markers.

#### SEM

SEM analysis was performed on a FEI electron microscope, using secondary electron beams with energies of 30 keV, on samples covered with a thin gold layer.

### *In vitro* biocompatibility of $\gamma$ -aminobutyric acid-silica network thin films

The biocompatibility of  $\gamma$ -aminobutyric acid-silica networks-based thin films with endothelial cells was asses by MTT Assay. Briefly, endothelial cells (EAhy926 cell line, ATCC) were grown on  $\gamma$ -aminobutyric acid-silica networks-based thin films substrates for 24 hours and viability measurements were done using a CellTiter96 Non-Radioactive Cell Proliferation MTT Assay kit (Promega,

Madison, USA), following the manufacturer's instructions. Cells proliferation was assessed through spectrophotometry measurements at 570 nm using a Mithras LB 940 apparatus (Berthold Technology, Germany). For revealing cell morphology and viability, fluorescent microscopy was assessed using a RED CMTPIX fluorophore (Life Technologies, Invitrogen, USA), a cell tracker for long-term tracing of living cells. The RED CMTPIX dye was added in the culture medium at a final concentration of 5  $\mu$ M and incubated 30 minutes for allowing the dye to penetrate the cells. Furthermore, the cells were washed with PBS and visualized by fluorescent microscopy. The photomicrographs were taken with a digital camera driven by the Axio-Vision 4.6 (Carl Zeiss, Germany) software.

### Biofilm development assay

The strains used in this study, *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were obtained from the American Type Culture Collection (ATCC, US). Strains from glycerol stocks were streaked on nutritive agar plates and colonies were allowed to develop for 24h at 37°C. Fresh colonies were used

to obtain a bacterial suspension of a 0,5 McFarland (corresponding to  $\sim 10^8$  CFU/mL) optical density in phosphate buffered saline (PBS). For assessing monospecific biofilms formation, 2 mL of nutritive broth were disposed in each well of a 6 wells plate, containing test ( $\gamma$ -aminobutyric acid-silica networks thin films coatings) and control (bare glass substrates). Bacterial inoculum consisted of a volume of 20 $\mu$ L from the PBS bacterial suspension, added in the 2mL of the nutritive broth. After a period of 24 h incubation at 37°C, the materials containing attached bacteria, were washed with PBS and transferred in a fresh well, containing 2mL sterile nutritive broth. Biofilm formation on the materials was assessed after 24, 48 and 72 h of incubation by the viable cell count (VCC) method. For this, after each time point, biofilm embedded bacteria cells were detached by vigorous vortexing for 30 seconds. PBS suspensions containing detached bacteria cells were subjected to serial dilutions and each dilution was seeded on nutritive agar. Experiments were performed in triplicate and repeated on at least three separate occasions.

## RESULTS AND DISCUSSIONS

Recently, we reported on the synthesis and characterization of  $\gamma$ -AA/SiO<sub>2</sub> nanoparticles with a diameter that not exceed 10 nm and with the pores diameter  $\sim 4.76$  nm (Grumezescu *et al.*, 2014a). Figure 1 presents the XRD pattern of  $\gamma$ -AA/SiO<sub>2</sub>. The pattern reveals a broad peak in the 15-35° range that can be assigned to silica with low degree of crystallinity.

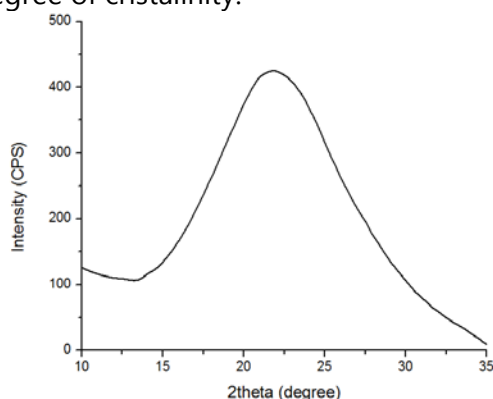


Figure 1. XRD pattern of gamma-AA/SiO<sub>2</sub>.

TEM images of the prepared gamma-AA/SiO<sub>2</sub> showed in figure 2 confirm the XRD

results, the size of nanoparticles being under 10 nm. All these results are in good agreement with our previous published paper (Grumezescu *et al.*, 2014a).

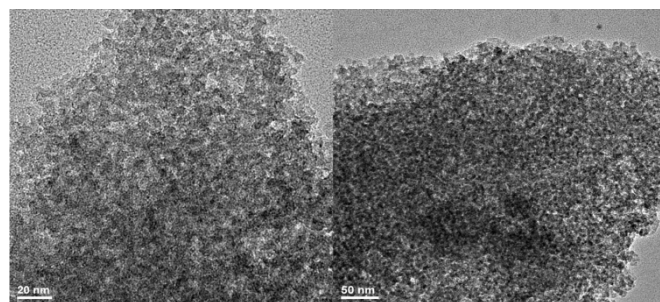


Figure 2. TEM images of gamma-AA/SiO<sub>2</sub>.

Infrared Microscopy was used to evaluate the integrity of functional groups of the prepared thin film by comparison with dropcast samples. For further investigations, the laser fluence at 400 mJ/cm<sup>2</sup> was selected as a compromise between the deposition rate and the stoichiometric transfer (Grumezescu *et al.*, 2014b). Figure 3 and 4 present

IR maps of dropcast and MAPLE thin films. The monitored band was characteristic to tetracycline ( $1616\text{ cm}^{-1}$ ). According to these figures it can be concluded that the thin films were successfully deposited without organic molecules degradation. In the FTIR spectra we observed that the

absorption bands are characteristics to silica ( $\text{Si-O}$ ,  $1057\text{ cm}^{-1}$ ) and to tetracycline ( $\text{C=O}$ ,  $1616\text{ cm}^{-1}$ ). The broad peak between  $3200\text{-}2900\text{ cm}^{-1}$  typical to the vibration of OH group is also visible for dropcast and MAPLE samples.

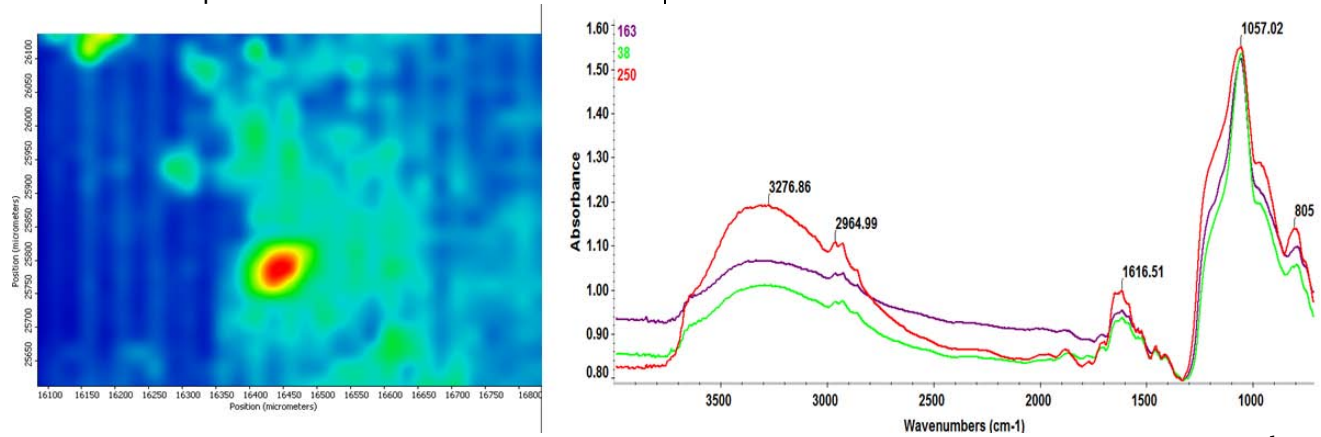


Figure 3. Second IR derivate mapping and IR spectra of gamma-AA/SiO<sub>2</sub>/TET dropcast sample: intensity distribution of  $1616\text{ cm}^{-1}$ .

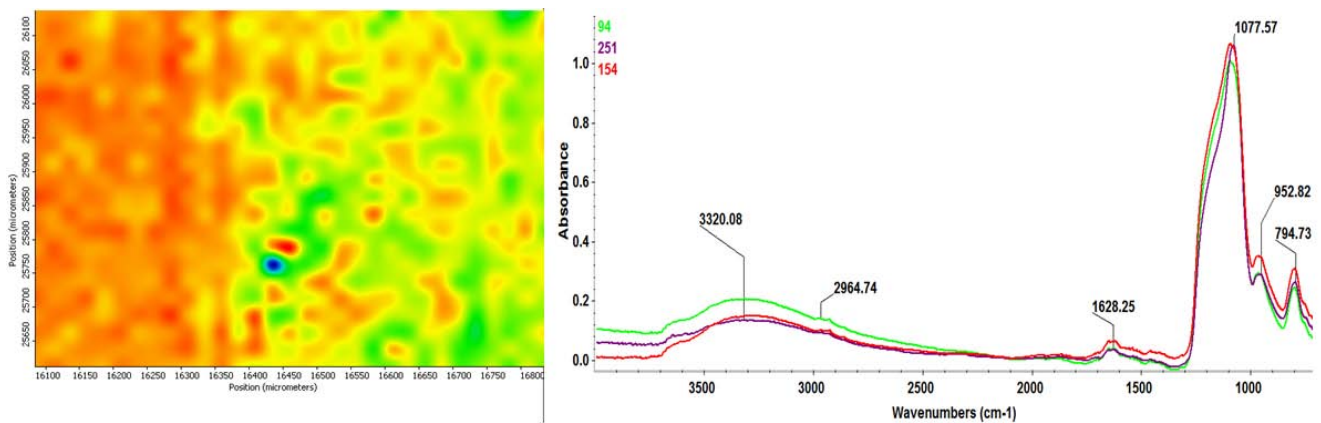


Figure 4. Second IR derivate mapping and IR spectra of gamma-AA/SiO<sub>2</sub>/TET thin film ( $F = 400\text{ mJ/cm}^2$ ): intensity distribution of  $1616\text{ cm}^{-1}$ .

Figure 5 shows the SEM images for the gamma-AA/SiO<sub>2</sub>/TET thin film. At low magnification, the thin film displays a homogeneous morphology with small rounded aggregates with the size within the range 200-300 nm uniformly distributed on the

surface. These results are in good agreement with our previous results (Grumezescu *et al.*, 2014b). The MAPLE processing of silica precursors/networks creates micro- and nano-particulates (Grumezescu *et al.*, 2014b).

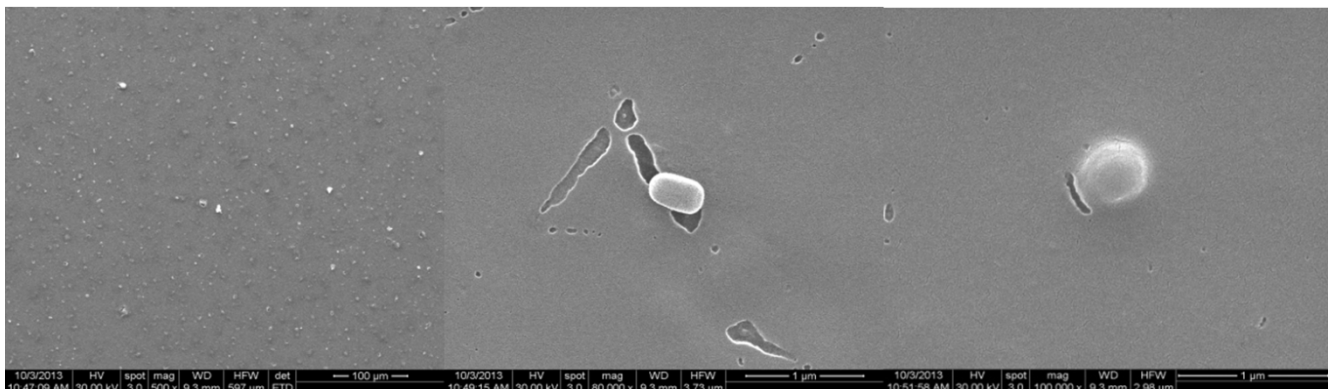
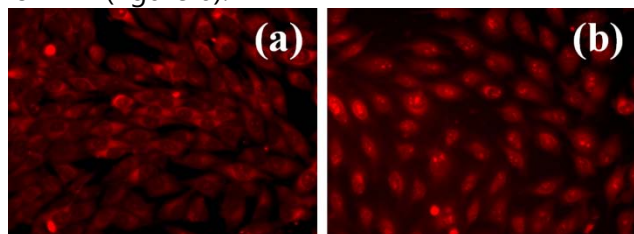


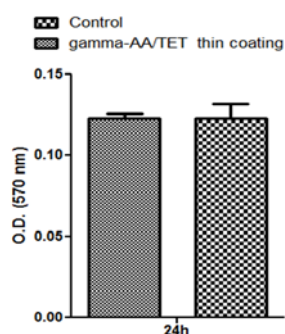
Figure 5. Typical SEM images of gamma-AA-SiO<sub>2</sub>/TET coatings.

Fluorescence microscopy assay demonstrated that  $\gamma$ -AA-SiO<sub>2</sub>/TET coatings does not modify the morphology and viability rate of endothelial cells grown on these modified surfaces for 24h (figure 6).



**Figure 6.** Fluorescence microscopy images of human endothelial cells developed on gamma-AA-SiO<sub>2</sub>/TET coatings (a) and control (b) surfaces for 24h.

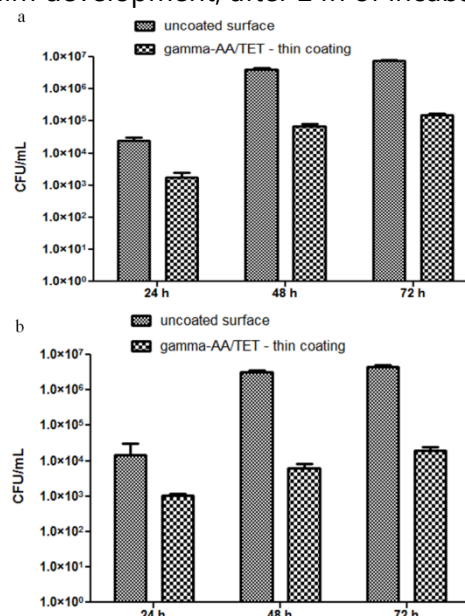
Furthermore, MTT assay evidenced that endothelial cells grown on  $\gamma$ -AA/SiO<sub>2</sub>/TET develop a normal proliferation rate and show an adequate metabolism rate, similar with the cells grown on the controls (bare glass substrates) (figure 7). These results demonstrate a good biocompatibility of this material with respect to human endothelial cells.



**Figure 7.** Graphic representation of quantitative evaluation of the human endothelial cells proliferation rates in the presences of gamma-AA-SiO<sub>2</sub>/TET coatings and control surfaces.

Even though the MAPLE fabricated thin coatings have no cytotoxic effects on cultured human cells, they proved a great effect on bacterial cell. The enhanced antimicrobial behavior may be explained by the fact that -AA-SiO<sub>2</sub>/TET thin coatings ensure the controlled release of the

antibiotic, enhancing its efficiency. Both *S. aureus* and *E. coli* biofilms development was impaired on the coated surfaces, the results revealing that the biofilm inhibitory effect is maintained along the bacterial incubation time. The most significant biofilm reduction was observed at the initial stages of biofilm development, after 24h of incubation.



**Figure 8.** Graphic representation of *S. aureus* (a) and *E. coli* (b) biofilm development on the control and nano-coated surfaces.

This result may be explained by the fact that the release of the antibiotic from the  $\gamma$ -AA-SiO<sub>2</sub> matrix is more pronounced in the early hours of biofilm development, or that the material inhibits bacterial initial attachments. We also observed for both *S. aureus* and *E. coli* a slight decrease in the antibiofilm efficiency of the fabricated coating during time, but the antimicrobial effect is still high enough to reveal a significant difference in the biofilms developed on the control and nanobiocoated surfaces (figure 8).

## CONCLUSIONS

Thin films based on silica networks/tetracycline were successfully transferred by MAPLE technique. SEM analysis reveals a good homogeneity of the sample with rounded particulates of 200-300 nm randomly distributed on the surface. The MAPLE thin films significantly

improved the resistance to microbial colonization, inhibiting the biofilm formation on both Gram positive (*S. aureus*) and Gram negative (*E. coli*) tested strains. These results correlated with their high biocompatibility highlight the possibility of using the  $\gamma$ -aminobutyric acid-silica network films

for the controlled local delivery of the therapeutic agents in low active doses that it may diminish the

occurrence of infection and rejection for implanted prosthetic devices.

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## Conflicts of Interest

The authors declare no conflict of interest.

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