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Preparations of Silver/Montmorillonite Biocomposite Multilayers and Their Antifungal Activity

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Abstract: In this study, the results about the influence of the surface morphology of layers based on montmorillonite (MMT) and silver (Ag) on antimicrobial properties are reported. The coating depositions were performed in the plasma of a radio frequency (RF) magnetron sputtering discharge. The studied layers were single montmorillonite layers (MMT) and silver/montmorillonite multilayers (MMT-Ag) obtained by magnetron sputtering technique with a different surface thickness. The resultant MMT-Ag biocomposite multilayers exhibited a uniform distribution of constituent elements and enhanced antimicrobial properties against fungal biofilm development. Glow-discharge optical emission spectroscopy (GDOES) analysis revealed the formation of MMT-Ag biocomposite multilayers following the deposit of a silver layer for an MMT layer that was initially deposited on a Si substrate. The surface morphology and thickness evaluation of deposited biocomposite layers were performed by scanning electron microscopy (SEM). A qualitative analysis of the chemical composition of thin layers was performed and the elements O, Ag, Mg, Fe, Al, and Si were identified in the MMT-Ag biocomposite multilayers. The in vitro antifungal assay proved that the inhibitory effect against the growth of Candida albicans ATCC 101231 CFU was more emphasized in the case of MMT-Ag biocomposite multilayers that in the case of the MMT layer. Cytotoxicity studies performed on HeLa cells showed that the tested layers did not show significant toxicity at the time intervals during which the assay was performed. On the other hand, it was observed that the MMT layers exhibited slightly higher biocompatible properties than the MMT-Ag composite layers.



Keywords: montmorillonite; silver; multilayers; antimicrobial activity; fungal biofilm

1. Introduction

Despite having a great immune system, we humans still cannot fight dangerous pathogens such as bacteria or fungi, and our contemporary civilization is still dependent on various other means of coping with them. Thus, we rely on modern-day technology to sustain this need. In this research, we pursued the study of experimental montmorillonite clays (also known as MMT) and their antimicrobial effectiveness, in an effort to combat or at least keep the pathogens at bay.

MMT clays have been shown to absorb heavy metal ions such as silver, copper, and zinc, thus acting as carriers, and the resulting clays have consequently been transformed into efficient antimicrobial fighters, mainly because the aforementioned metal ions have been used for sterilizing actions for centuries. MMT-AgNO₃ turned out to be extremely efficient against pathogenic bacteria such as Escherichia coli and Staphylococcus aureus. This combo displayed 99% efficacy against the bacterial growth mentioned above, as well as having an extraordinary blood and tissue biocompatibility by manifesting low cytotoxicity and low hemolytic activity [1]. Moreover, by adding different-sized silver nanoparticles to MMT, the antimicrobial activity against Gram-positive (i.e., S. aureus) and Gram-negative (i.e., E. coli and Klebsiella pneumoniae) bacteria was studied, resulting in higher efficacy with the modification in size of Ag nanoparticles by case [2]. In addition, it was observed that positively charged ions of silver (Ag⁺) can attract the membrane of Gram-negative bacteria to the surface of the experimental MMT clay, thus destroying the bacteria efficiently [3]. The use of silver-loaded montmorillonites (Ag-MMT) was also shown to improve the shelf life of fresh fruit salads by inhibiting the microbial growth. It also improved the sensorial qualities of the fresh-cut fruit samples stored in an active packaging based on Ag-MMT coating by leading to longer and better preservation, thus representing a viable solution for the packaging industry against the fast decaying of fresh fruits [4]. Likewise, researchers noted that Ag-MMT had a slow-release property, besides the already known antibacterial efficiency, thus making it a great low-cost material for different purposes, whether it be the medical field or food-related industries [5]. A strong antimicrobial effect was noted when Zn²⁺-Ce³⁺ were added to MMT, thus demonstrating a great efficacy in combating a wide spectrum of harmful pathogens like E. coli, S. aureus, Candida albicans, and Mucor, due to the synergistic effect of Zn and Ce [6,7]. Active biofilms based on chitosan, glycerol, and MMT-CuO exhibited significant antimicrobial activity against the foodborne pathogenic bacteria S. aureus, Bacillus cereus, E. coli, and Pseudomona aeruginosa with 99% effectiveness. Moreover, the antibacterial and antifungal properties of silver, copper, and zinc montmorillonites against E. coli CCM 3988, Pleurotus ostreatus, and Pycnoporus cinnabarinus were also demonstrated [6]. Furthermore, studies have emphasized that poly(dimethylsiloxane) (PDMS)/montmorillonite-terbinafine hydrochloride (PDMS/OMMT) thin film also exhibited distinctive microbial inhibition zones against various microorganisms [8], and a strong inhibition effect against the fungal strain C. albicans.

Wang et al. [9] found that MMT nano-form clay was found to enhance the thermal stability and also the sturdiness and elastic modulus of polymer matrices, the beneficial effect being directly proportional to the added quantity. Moreover, MMT-Cu(II) demonstrated its efficacy in vitro against *E. coli* and *Salmonella choleraesuis* by inhibiting their growth, more specifically by damaging the bacterial cell wall, by causing bacterial enzyme leakage, and by inhibiting the bacterial respiratory metabolism. These promising results may lead to new non-toxic antibiotic alternatives that can act against dangerous pathogens found in the gastrointestinal tract of animals destined for human consumption such as pigs [10]. Researchers have also remarked the antibacterial potential of chlorhexidine acetate–montmorillonite (CA-MMT) to strongly inhibit the growth of *S. aureus* and *P. aeruginosa*, and it was concluded that MMT clays can act as potential drug delivery carriers with controlled-releasing properties [11]. CA-MMT organoclay displayed potent antimicrobial activity that can last up to one year. The antibacterial activity was enhanced as the CA loading increased, thus making it suitable for the creation of novel antibacterial materials [12]. Likewise, the efficacy of curcumin-loaded MMT was demonstrated against *Streptococcus mutans* in preventing the bacterial proliferation to take place on experimental dental models [13]. It was also shown that MMT clays can be successfully used as reinforcing dental fillers, thereby making them a great solution as adhesives between dentin and restorative resin [14]. Clay minerals have been bringing important benefits to people and society. Montmorillonite, an important mineral clay type, has been gaining recent interest in being used in polymer matrices, due to its versatile characteristics—especially the one about being a carrier for those materials possessing antimicrobial properties. Recently, nanocomposite layers with MMT fillers were generated by oxidative polymerization [15] or sol–gel chemical synthesis methods [16].

Currently, different methods such as magnetron sputtering, pulsed laser deposition, biomimetic crystallization, electrospinning, or sol–gel are used for generating biocompatible multicomponent coatings on metallic and polymeric substrates for biomedical applications [17]. Among these methods, radio frequency (RF) magnetron sputtering has proven its capabilities and benefits in generating multicomponent coatings, as the layers present high purity, uniform thicknesses, and good adhesion to the substrate [17,18]. Coatings of biomaterials with antimicrobial properties deposited on orthopedic prostheses are widely used for increasing the osteointegration and bone bonding rate of those devices [17].

In this paper are presented the results obtained about the MMT and MMT-Ag layers deposited on Si substrates by radio frequency magnetron sputtering discharges. Furthermore, the purpose of this research was to study the influence of the morphology of montmorillonite (MMT) and silver (MMT-Ag) layers on antimicrobial properties. The thickness evaluation and surface morphology of the layers were studied by scanning electron microscopy (SEM). Energy-dispersive X-ray spectroscopy (EDS) analysis was used for chemical analysis and evaluation of the distribution of constituent elements in composite layers. A qualitative analysis of the chemical composition of thin layers was performed by glow-discharge optical emission spectroscopy (GDOES).

2. Materials and Methods

2.1. MMT and MMT-Ag Composite Layers

The coating depositions were performed in the plasma of an RF magnetron sputtering discharge in Ar gas flow in a single run, using an RF magnetron source of 2 inches in diameter (K.J. Lesker Company, Hastings, UK) and an MMT sputtering target. The 2-inch diameter target was prepared by mechanical pressing of the MMT powder (montmorillonite K10 purchased from Alfa Aesar and used as received; Karlsruhe, Germany) in air for few minutes. The applied RF power was 90 W, while the magnetron source-grounded substrate holder distance was 8 cm. The Ar working pressure was set to 6 $\times 10^{-3}$ mbar (base pressure $\approx 10^{-5}$ mbar) and the Ar gas flow was about 12 mln/min. The deposition time was 2 h. A detailed experimental setup of the deposition chamber is presented in [18].

The Ag layers were deposited in an RF magnetron sputtering discharge on the surface of the MMT layers by using a 2-inch diameter Ag sputtering target acquired from K.J. Lesker Company (Hastings, England) under the following conditions: 100 W RF power, 1×10^{-2} mbar Ar working pressure, 22 mln/min gas flow, and magnetron source-grounded substrate holder distance of 8 cm. The deposition time was about 50 s.

2.2. Characterization Methods

A Quanta Inspect F50 FEG scanning electron microscope with 1.2 nm resolution, equipped with an energy-dispersive X-ray (EDX) analyzer (resolution of 133 eV at MnK α , Thermo Fisher Scientific, Waltham, MA, USA) was used to analyze the morphology and the chemical composition of the samples. Using glow-discharge optical emission spectroscopy (GDOES), we investigated the distribution of the chemical elements that are characteristic of the MMT into the MMT and MMT-Ag composite-based

layers deposited on the Si substrates by magnetron sputtering discharge. The working parameters of the GD-Profiler 2 (Horiba Company, Longjumeau, France) for the analysis of these composite layers were as follows: 650 Pa, 35 W RF power impulse mode at 1 kHz, and a duty cycle of 0.25. The MMT powders used to obtain the targets for the MMT layers were pressed into pellets and investigated using a laser ablation inductively coupled plasma mass spectrometer (LA-ICP-MS) to perform an elemental analysis. The investigations were performed as previously described by Motelica-Heino and Donard [19], with an XR Thermo Fisher Scientific instrument (Waltham, MA, USA) combined with a UV laser probe laser ablation sampling device (Teledyne CETAC Technologies, Omaha, NE, USA). An artificial certified glass, NIST-610, was used for the calibration. The measurements were done in triplicate.

The surface morphology of the films was analyzed by atomic force microscopy (AFM), in non-contact mode, with the help of an NT-MDT NTEGRA Probe NanoLaboratory system (NT-MDT, Moscow, Russia). A silicon NT-MDT NSG01 cantilever coated with a 35 nm gold layer was used, having a tetrahedral tip, with a curvature radius of 35 nm and height of 14–16 μ m. The AFM micrographs were acquired on surface areas of 50 × 50 μ m², and the root mean square roughness (R_{RMS}) was inferred.

The crystalline status of the source powder and thin films was investigated by X-ray diffraction (XRD). The powder was analyzed in Bragg–Brentano geometry by using a Bruker D8 Advance diffractometer (Bruker, Karlsruhe, Germany) with Cu K_{α} (λ = 1.5418 Å) radiation, equipped with a high efficiency LynxEyeTM 1D linear detector. The thin films were examined in grazing incidence (GIXRD) geometry, with Rigaku SmartLab 3 kW equipment (Tokyo, Japan), using CuK_{α} radiation (λ = 1.5418 Å) and an incidence angle of 1.5°. The patterns were acquired in the 2 θ range of 5–70°, with a step size of 0.04°.

The chemical structure of the source material and film was analyzed by Fourier-transform infrared (FTIR) spectroscopy in attenuated total reflectance (ATR) mode, using a PerkinElmer Spectrum BX II spectrometer (Waltham, MA, USA) equipped with a PIKE MIRacle ATR head with a diamond–ZnSe crystal plate. The spectra were recorded in the range of 4000–535 cm⁻¹ wave numbers, at a resolution of 4 cm⁻¹, and represent the average of 64 individual scans.

2.3. In Vitro Antifungal Assays

The antifungal activity of the MMT and MMT-Ag biocomposite layers was assessed using the reference fungal strain C. albicans ATCC 10231 from the American Type Culture Collection (ATCC, Manassas, VA, USA). The experiments were performed with fungal suspensions of approximately 1.5×10^6 colony forming units (CFU)/mL obtained from 15 to 18 h bacterial cultures as previously reported in Iconaru et al. [20]. The qualitative assay of the fungal biofilm development, after incubation at 24, 48, and 72 h, was assessed by visualization of the adherent fungal cells on the substrates using scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). For SEM investigation, the MMT and MMT-Ag biocomposite layers were removed from the *C. albicans* ATCC 10231 culture medium after 72 h of incubation, washed with sterile saline solution, then fixed using cold methanol, and prepared for scanning electron microcopy (SEM) visualization. For this purpose, for CLSM visualization and after incubation at different time intervals (24, 48, and 72 h), the MMT and MMT-Ag biocomposite layers were washed with sterile saline buffer (PBS) in order to remove the unattached fungal cells and then fixed with cold methanol and stained in the dark with propidium iodide (PI) for 10 min at room temperature (5 µg/mL PI solution in distilled water). After the staining, the PI excess was eliminated using filter paper. The samples were examined and the specimens were examined directly after staining. The visualization was performed both in reflection and fluorescence modes using a Leica TCS-SP confocal microscope (Wetzlar, Germany), equipped with a PL FLUOTAR ($40 \times NA 0.7$) objective, and an Ar ion laser with a laser line at 488 nm [21–23].

The quantitative assay of the antifungal activity of MMT and MMT-Ag biocomposite layers was carried out by an adapted method (E2149-10; ASTM International) [24,25], previously described by

Predoi et al. [26]. The experiments were performed in triplicate and the results of the results were expressed as mean \pm SD.

2.4. In Vitro Cytotoxicity Assays

The biocompatible properties of the MMT and MMT-Ag biocomposite layers were investigated by cytotoxicity assays using the HeLa cell line. The in vitro studies regarding the influence of MMT and MMT-Ag biocomposite layers against the development of HeLa cells were conducted at three time intervals (24, 48, and 72 h). For the cytotoxicity assays, HeLa cells were grown in Dulbecco's modified Eagle's medium at 37 °C. Afterwards, MMT and MMT-Ag biocomposite layers were incubated with the cells. The toxicity of the MMT and MMT-Ag biocomposite layers against HeLa cells was done using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. The in vitro cytotoxicity assays were done in triplicate.

3. Results and Discussion

Characteristic AFM images for the surface morphology of the MMT layer and MMT-Ag composite multilayers are presented in Figure 1a,b. The MMT layer, formed of large grains (\approx 15 to 30 µm) with salient boundaries, were found to be rather rough, having R_{RMS} values of \approx 280 nm. However, the roughness is known to play a prominent role in the bone–implant contact and biological response, improving on the one hand the implant retention in situ, and inducing on the other hand an increase in cell viability and proliferation [27–30]. The simple MMT layer seemingly had a denser morphology (Figure 1a), whereas the MMT-Ag composite multilayers, composed of elongated micro-crystals (Figure 1b) with assorted sizes in the range of \approx 4 to 8 µm, had changed dramatically its general surface appearance and roughness (R_{RMS} \approx 510 nm).

Figure 1c,e displays comparatively the XRD and GIXRD of the MMT powder and MMT layer and MMT-Ag composite multilayers, respectively. The source MMT powder is well crystalized and is constituted of a major montmorillonite-type compound and minor SiO₂ (quartz) phase (Figure 1c), as reported in the literature [31]. The MMT layer is amorphous at the sensitivity limit of the employed XRD apparatus, as indicated by the shallow and broad halo positioned in the 2 θ from \approx 40 to 60° angular region (Figure 1e inset). The top Ag layer, deposited in a subsequent step on the surface of the MMT layer, is composed of a cubic silver phase (Figure 1e). The average crystallite size was estimated from the full width at half maximum (FWHM) of the 111 XRD line by applying the Scherrer equation [32]. The line width was corrected for instrumental broadening using a corundum NIST SRM 1976b standard reference. An average crystallite size of \approx 450 nm was calculated. Consequently, the above observed grains are composed of \approx 9to 18 crystallites.

FTIR-ATR analyses indicated the chemical structure of the MMT powder and the derived layers (Figure 1d,f). The MMT powder elicited all the IR absorption bands that are characteristic of a montmorillonite compound (Figure 1d), in both fingerprint (i.e., symmetric stretching of Si–O bonds (\approx 797 cm⁻¹), vibrations of Al–Al–OH and Si–OH (\approx 916 cm⁻¹), and asymmetric stretching of Si–O bonds (\approx 1020 and \approx 1167 cm⁻¹)) and functional group (bending (δ) and stretching (ν) of water molecules (at \approx 1631 and \approx 3405 cm⁻¹, respectively) and stretching of inner hydroxyl groups coordinated to octahedral cations) regions [31,33]. The spectrum of the layer had a broader allure (Figure 1f), indicative of a disordered material (in good agreement with the GIXRD observations). A first observation is that the IR vibration bands connected to the water or hydroxyl are virtually extinct. This is to expected for films deposited by physical vapor deposition processes, in vacuum or at low inert gas pressures [34]. Furthermore, a shift (from \approx 1020 cm⁻¹ to \approx 1035 cm⁻¹) is noticed for the first ν_{as} Si–O band. This band is known to be highly sensitive to both short-range order and compositional changes occurring in the vicinity of the silicate tetrahedra environment [31]. This IR band position is distinctive for a vitreous silica-based structure deposited by magnetron sputtering [35,36]. The supplemental bands peaking for both source material and thin film in the \approx 2800 to 3100 cm⁻¹ region appertain to various stretching



vibrations of C–H bonds [37], and suggest the presence of ordinary carbonaceous residues on the samples' surface as result of measurement, handling, and/or storage in normal ambient conditions [38].

Figure 1. Characteristic atomic force microscopy (AFM) images of the (**a**) montmorillonite (MMT) layer and (**b**) silver/ montmorillonite (MMT-Ag) composite multilayers, collected on areas of $50 \times 50 \ \mu\text{m}^2$. X-ray diffraction (XRD) patterns, recorded in (**c**) Bragg–Brentano and (**e**) grazing incidence ($\alpha = 1.5^\circ$) geometry, of the (**c**) MMT powder and (**e**) MMT layer and MMT-Ag composite multilayers, respectively. Fourier-transform infrared spectroscopy in attenuated total reflectance (FTIR-ATR) spectra of the (**d**) MMT powder and (**f**) MMT layer and MMT-Ag composite multilayers.

A widely used spectroscopic method for analyzing the elemental composition of thin layers as well as their chemical structure is glow-discharge optical emission spectroscopy (GDOES). The GDOES depth profiles of thin layers deposited on metallic or nonmetallic substrates are obtained by measuring the emission intensities of each chemical element of the sample sputtered in the RF glow discharge as a function of layer thickness or sputtering time.

Figure 1a,b shows the depth profiles of MMT and MMT-Ag-based composite layers, respectively, obtained in the RF magnetron sputtering discharge. All the chemical elements that are characteristic of the MMT, such as Na, Ca, Al, Mg, Si, O, and H, can be found in the layers. In Figure 1a, the similar behavior of the Na and Ca depth profile curves and of the Al and Mg depth profile curves can be observed, indicating that these elements are coupled into chemical bonds. In addition, the Si and O depth profile curves simultaneously appeared. In Figure 1b, a not so clear delimitation between the Ag

and MMT layers can be observed. It seems that, during the deposition process of the Ag layer, some of the Ag atoms diffused into the MMT composite-based coating previously deposited on the Si substrate by the magnetron sputtering technique. However, the spectrum from Figure 1b shows the formation of a compact Ag layer on the surface of MMT composite-based thin film. It is important to notice the absence of the oxygen depth profile simultaneously with the Ag depth profile, suggesting that the Ag film formed on the surface of the MMT composite-based layer is free of oxides.

The MMT and MMT-Ag-based composite layers/Si substrate interfaces can be evidenced by the Si depth profile curve evolution as a function of thickness. In both graphs from Figure 2, the decrease in the Na, Ca, Al, Mg, Si, O, and H depth profile curves is accompanied by an increase in the Si depth profile curve but not in a sharp, knife-like manner specific to multilayer coatings [39]. Such a behavior may be attributed to the diffusion of all the sputtered chemical elements (Na, Ca, Al, Mg, Si, O, and H) that compose the MMT multicomponent target into the Si substrate, during the magnetron discharge deposition process. As the Si substrate has a mirror-like surface and the GD Profiler operating conditions were chosen for providing a flat crater bottom, the spectra from Figure 1a,b indicate the formation of composite layers of MMT.



Figure 2. The glow-discharge optical emission spectroscopy (GDOES) depth profiles of the (**a**) MMT layer and (**b**) MMT-Ag composite multilayers.

To characterize the surface morphology of the MMT and MMT-Ag layers, an SEM analysis was carried out. The SEM micrographs of the MMT layer and MMT-Ag multilayers are shown in Figure 3a–c. The temperature of the Si substrate during the layer deposition in the plasma of an RF magnetron sputtering discharge was constant (around 150 °C).

The image of the MMT layer's surface at two magnifications is presented in Figure 3a,b. It can be seen that the MMT layer is uniform without cracks. The formed MMT-Ag biocomposite multilayers are uniform and present a better surface structure. It is obvious that the growth of the MMT-Ag composite multilayer was different from that of the MMT layer by the diffusion rate of silver atoms on the MMT surface in the deposition process. Furthermore, when the Ag and MMT layers were alternately deposited, the resulting MMT-Ag composite multilayers were more stable due to the hydrogen-bonding effects or the completion of the vacancies that were formed when the layers were deposited individually, thereby blocking the H₂O permeation [40–42].

Figure 4 shows the thickness of the (a) MMT layer and (b) MMT-Ag multilayers deposited on the Si substrate. The thickness of the MMT layers was 635.9 nm (Figure 4a), whereas the thickness of the MMT-Ag multilayers was 1122.60 nm. Figure 4b shows the formation of three layers following the silver coating of the MMT layer initially deposited on the silicon substrate. The thickness of the first layer formed on the Si substrate was 799.91 and is a layer of MMT. The second layer (MMT-Ag) was formed by the diffusion of silver atoms into the initially deposited MMT layer and has a thickness of

287.05 nm. The third layer is a silver layer with a thickness of 85.64 nm that uniformly covers the entire surface evenly. The second and third layers were formed during the deposition of the Ag layer over the MMT layer initially deposited on the Si substrate. Energy-dispersive X-ray spectroscopy (EDS) was performed for an analysis of the elemental composition of the layer. The elements O, Ag, Mg, Fe, Al, and Si were identified in the EDS spectrum (Figure 5a). The uniform distribution of constituent elements in the MMT-Ag biocomposite layers was also observed (Figure 5b–g).



Figure 3. SEM images of the (**a**,**b**) MMT layer and (**c**,**d**) MMT-Ag composite multilayers.



Figure 4. Cross-sectional scanning electron microscopy (SEM) image of the (**a**) MMT layer and (**b**) MMT-Ag composite multilayers.



Figure 5. (a) Energy-dispersive X-ray spectroscopy (EDS) spectrum of MMT-Ag composite multilayers; (b–g) Uniform distribution of constituent elements in MMT-Ag composite multilayer.

A quantitative elemental analysis was also performed on the MMT powders using the LA-ICP-MS technique. The results of the studies revealed that MMT powders consisted of Na ($2.20 \pm 0.3 \text{ wt }\%$), Ca ($4.96 \pm 0.3 \text{ wt }\%$), Mg ($2.90 \pm 0.8 \text{ wt }\%$), Al ($17.42 \pm 0.4 \text{ wt }\%$), Si ($57.52 \pm 0.5 \text{ wt }\%$), and Fe ($11.04 \pm 0.8 \text{ wt }\%$). The results obtained are in agreement with the GDOES results obtained in our study and are also comparable with previous reported studies [43].

Recently, montmorillonite clays were reported as being widely used both in the biomedical field and in waste water remediation as adsorbents of organic and inorganic contaminants [44]. In this context, their biological properties were also taken into consideration and studied worldwide. In their in vivo studies, Li et al. [45] emphasized that MMT did not affect the mortality of Sprague Dawley (SD) rats when they were fed specific doses of MMT. Moreover, the authors proved that the MMT did not induce any changes in cell morphology. Furthermore, pharmacology studies emphasized that MMT has the ability to adsorb to bacterial cells like *E. coli* and *S. aureus*, and to immobilize cell toxins [45–47].

In this study, the antifungal activity of MMT and MMT-Ag composite layers was investigated using the reference fungal strain *C. albicans* ATCC 101231. The in vitro antifungal assay was conducted

at three different time intervals (24, 48, and 72 h) using an adapted method of the standard test method for the determination of the antimicrobial activity under dynamic contact conditions. The results of the quantitative studies are presented in Figure 6. The quantitative assays highlighted that both MMT and MMT-Ag composite layers inhibited the development of colony forming units (CFUs) even after 24 h of incubation in comparison to the control. The results of the in vitro antifungal assay demonstrated that the inhibitory effect against the development of *C. albicans* CFUs was more pronounced in the case of MMT-Ag composite layers than in the case of MMT layers. In addition, as it can be seen, the inhibitory effect was strongly influenced by the incubation time. In the case of MMT-Ag composite layers, after a 72 h incubation time, the *C. albicans* cell development is almost insignificant related to the control, suggesting a fungicidal effect of the investigated sample. This behavior is attributed to the presence of silver ions in the composite layers and also to their controlled released over time. A strong inhibitory effect against the development of *C. albicans* was also noticed after 48 h in the case of MMT-Ag samples. The calculated *p*-value for the antimicrobial assays was equal to 0.857. This difference is considered not statistically significant judging by conventional methods.



Figure 6. Antifungal effect of MMT and MMT-Ag composite layers against C. albicans ATCC 10231.

Although the antifungal activity of MMT layers was smaller than the one exhibited by MMT-Ag composite layers, these results are also very important and prove that MMT layers can be used as antifungal agents. The antifungal activity exhibited by the MMT layers can be attributed to the metal ions from the composition of MMT such as magnesium and aluminum. The results obtained are in good agreement with several existing studies related to the antibacterial activity of MMT and MMT-based materials and thin films. In their study, regarding "the wettability, mechanical and antimicrobial properties of polylactide/montmorillonite nanocomposite films", Rapacz-Kmita et al. [48] reported that a bactericidal effect against Gram-positive bacteria S. aureus and Enterococcus faecalis as well as against a lactose-positive bacterium from the Enterobacteriaceae family was obtained for polylactide/montmorillonite nanocomposite films. Moreover, the antibacterial activity of modified MMT clays against bacterial strains such as E. coli, S. aureus, and Enterobacter cloacae was also reported by Kenawy et al. [49]. The results obtained by the antifungal assay are in agreement with other reported studies regarding the antimicrobial activity of MMT and MMT-based composites [48,49]. In their study, Malachová et al. [6] also demonstrated that MMTs enhanced with various metallic ions such as Cu, Zn, and Ag have shown promising antimicrobial properties. Moreover, their results [6] emphasized a strong antifungal effect against P. cinnabarinus and P. ostreatus fungal strains and also that the antifungal properties observed in the case of zinc, copper, and silver ion MMTs were unquestionably better than the ones obtained in the case of MMT samples. Additionally, Malachová et al. [6] investigated the antibacterial effect of the MMTs enriched with different metal ions against the *E. coli* strain and their results demonstrated that, because montmorillonite absorbs heavy metal ions and can act as their

carrier, these types of materials could be used in the development of antibacterial and antifungal agents. The development and adherence of the *C. albicans* ATCC 10231 fungal cells on the surface of MMT and MMT-Ag composite multilayers after 72 h of incubation were evaluated by visualization using scanning electron microscopy (SEM). The images obtained by SEM of the MMT and MMT-Ag composite layers after 72 h incubation with fungal suspensions of *C. albicans* ATCC 10231 are presented in Figure 7. SEM studies revealed that the morphology of the adhered cells are specific to *C. albicans* fungal cells presenting oval-shaped cells and having sizes in the range of 2.43 µm to 5.38 µm.



Figure 7. SEM images of *Candida albicans* ATCC 10231 cell development on (**a**) MMT and (**b**) MMT-Ag composite layers after 72 h of incubation.

Moreover, the SEM investigations highlighted that the surfaces of MMT and MMT-Ag composite layers prevented fungal cell adhesion and that, after 72 h of incubation, only a few *C. albicans* fungal cells were present on the surface of the composite layers. Furthermore, the results of the SEM visualization emphasized that MMT-Ag composite layers had a more pronounced inhibitory effect against *C. albicans* cell development than the MMT composite layers.

The results of the SEM investigations showed that the MMT and MMT-Ag composite layers demonstrated excellent antifungal properties against the *C. albicans* ATCC 10231 fungal strain. Moreover, these results are in good agreement with the quantitative antifungal assays, which evidenced that the MMT-Ag composite layers were more effective against the development of *C. albicans* fungal cells than were the MMT composite layers.

Furthermore, the development and adhesion of *C. albicans* cells on the surface of MMT and MMT-Ag composite layers after different incubation times (24, 48, and 72 h) were also examined by confocal laser scanning microscopy (CLSM) and are presented in Figure 8a–f. The CLSM visualization highlighted that cells adhered to the surfaces of MMT and MMT-Ag composite layers presented a morphology with a typical "ovaloid" shape specific to *C. albicans* fungal cells and had sizes ranging from 2.49 μ m to 5.66 μ m. More than that, the CLSM studies revealed that the development of the fungal cells was inhibited even after 24 h of incubation by both samples.

In addition, the CLSM visualization emphasized that the MMT-Ag composite layers had a better inhibitory effect against fungal cell development, and that for all the tested intervals the MMT-Ag composite layers presented a fungicidal effect against the development of C. *albicans* biofilms. As can be seen in Figure 8d–f, no biofilm formation was observed in the case of MMT-Ag composite layers at any of the tested intervals. The images also reflect that the MMT composite layers presented a good inhibitory effect against the development of *C. albicans* fungal cells for all tested intervals. Moreover, the CLSM visualization emphasized that the inhibitory effect is dependent both on the tested surface and on the incubation time.



Figure 8. Confocal laser scanning microscopy (CLSM) images of *C. albicans* ATCC 10231 cell development on (**a**–**c**) MMT composite layers after 24, 48, and 72 h of incubation and (**d**–**f**) MMT-Ag composite layers after 24, 48, and 72 h of incubation.

Our results were in good agreement with the previous studies [50,51] that showed that MMT can play an important role in the realization of biomaterials, MMT being a support for the anchoring of transition metal complexes and as adsorbents for cationic ions. The antifungal activity of the MMT layer and MMT-Ag biocomposite multilayers showed a strong antifungal activity against *C. albicans* ATCC 10231 fungal strains. These results complement the previous research that showed that silver nanoparticles in MMT/chitosan exhibit strong antibacterial activity against Gram-positive and Gram-negative bacteria [52]. Moreover, the results showed that the antifungal properties of MMT layers can be modified when their surface is modified by depositing a silver layer. To estimate the antifungal effects of MMT-Ag biocomposite multilayers, further studies on other types of fungi are needed which may lead to the potential expansion of their applications in the medical field such as surgical devices. These studies are needed the more so as the incidence of fungal infections has increased greatly, becoming a difficult issue to manage as antibiotic resistance increases with the increasing diversity of etiological agents.

Complementary biological studies regarding the cytotoxicity of MMT and MMT-Ag composite layers were also conducted using the HeLa cell line. Therefore, the influence of the MMT and MMT-Ag composite layers on the development of HeLa cells was investigated at different time intervals. In order to evaluate the toxicity of the MMT and MMT-Ag layers towards HeLa cells, an MTT quantitative assay was conducted and HeLa cell viability was determined after 24, 48, and 72 h of incubation in the presence of MMT and MMT-Ag layers. The results obtained from the cytotoxicity studies revealed that none of the tested layers presented any significant toxicity against the HeLa cells at any tested time interval (Figure 9). The results of the quantitative MTT assays revealed that, in the presence of MMT layers, HeLa cell viability decreased from 96% at 24 h to 94% at 48 h and to 92% after 72 h of incubation.

The MTT quantitative studies highlighted that, after 24 h of incubation time with the MMT layers, HeLa cell viability did not undergo any major modification in comparison to the control cell culture. Moreover, the same behavior was observed in the case of HeLa cells incubated with MMT layers after 48 and 72 h. Similar results were obtained in the case of HeLa cell incubation with MMT-Ag composite layers for 24, 48, and 72 h. The results of the MTT assay emphasized that HeLa cell viability decreased slightly from 93% to 90% and 88% after 24, 48, and 72 h, respectively, of incubation with MMT-Ag composite layers. The results highlighted that there is a correlation between the incubation time and



the small cytotoxic effects presented by the tested samples. Moreover, the MTT suggested that the MMT layers exhibited slightly better biocompatible properties than the MMT-Ag composite layers.

Figure 9. MMT and MMT-Ag layers influence on HeLa cell viability at different incubation times.

These results are in good agreement with previous studies that reported MMT as being a non-toxic and bioinert material [53–55]. Moreover, Lal et al. [55] concluded that their Mt-insulin-PLGA nanocomposites obtained using the double emulsion solvent evaporation method were highly biocompatible and did not show any toxicity against human embryonic kidney HEK-293. Furthermore, Corrales et al. [56] demonstrated in their study that PCL and PCL/MMT films are highly biocompatible. They reported that both poly(ε -caprolactone) (PCL) and poly(ε -caprolactone)/ montmorillonite (PCL/MMT) films allowed the culture of murine L929 fibroblasts on their surface and the cells presented a high percent of viability, very scarce signs of cell apoptosis, and no plasma membrane damage. The study of Corrales et al. emphasized that the presence of MMTk10 or MMT did not significantly affect the viability of L929 fibroblasts and therefore concluded that these types of materials may be useful candidates for tissue engineering. Therefore, being in agreement with the literature, the results obtained in this study regarding the biological aspects of MTT and MTT-AG composite layers showed that both types of layers exhibited high biocompatibility and also significant antifungal properties, making them ideal candidates for the development of future antimicrobial agents.

4. Conclusions

In summary, MMT and MMT-Ag layers were prepared using the magnetron sputtering technique. The resultant layers exhibit a uniform distribution of constituent elements. The thickness measurements show that the MMT-Ag biocomposite layers have a greater thickness. EDS spectra of MMT-Ag biocomposite layers revealed the presence of Ag in the thin layers and thus the formation of biocomposite layers.

The antifungal assays confirmed that both MMT and MMT-Ag biocomposite layers demonstrated good antifungal activity at all tested time intervals. Furthermore, the quantitative assay emphasized that, after 72 h of incubation, the MMT-Ag biocomposite layers exhibited fungicidal activity against *C. albicans* cell development. Furthermore, the visualization of *C. albicans* cell development and their adherence to the surface of the MMT and MMT-Ag biocomposite layers using SEM and CLSM confirmed the antifungal activity of the tested samples. Moreover, the CLSM images highlighted that the MMT-Ag composite layers presented a fungicidal effect against *C. albicans* biofilm development. The in vitro cytotoxic assays revealed that the MMT and MMT-Ag composite layers did not exhibit any significant toxicity against HeLa cell development. The developed nanocomposite thin layers may

have an excellent synergistic effect of biocompatible MTT and antimicrobial silver ions. Therefore, these types of materials indicate a promising potential for being used in the development of biomedical applications in the near future.

The results obtained in this study may contribute to the understanding of clay antimicrobial activity when silver ions are also involved. This understanding of the mechanisms and synergies involved here may help in the design and development of new antimicrobial systems. These types of materials have promising biocompatible and antimicrobial properties and may be considered for use as coatings both for medical prostheses and dental implants.

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