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Ph.D. Domain: *Chemical Engineering*

Ph.D. Thesis Summary

Bioactive antibacterial coatings for bone tissue regeneration

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Keywords: *antibacterial coatings, Matrix-Assisted Pulsed Laser Evaporation, hydroxyapatite, magnesium phosphate, silver nanoparticles, nanotechnology*

Contents

Abstract

Currently, the appearance of infections in medical setting represents one of the biggest concerns, especially because of their high antibiotic resistance. In some cases, they can become lifethreatening or can lead to serious complications. The design and development of effective and controlled tactics to overcome antibiotic resistance and to optimize therapeutic effect is a must. Advancements in nanotechnology offered the possibility to create next-generation antibacterial coatings. The main scope of such coatings is to be multifunctional, to possess high stability and to combine multiple biocidal effects to address as many parameters as possible in the infection appearance process. In the past decades, research has been focused in this domain so various strategies were investigated. The aim of this thesis was to address this challenge by design and characterization of nanostructured antibacterial coatings capable of bacterial attachment inhibition, together with preventing spread and growth on substrates and, in the same time, to enhance bone regeneration process. Novel techniques were used in the fabrication of these coatings which were further characterized by multiple advanced methods and subjected to in-vitro testing and antibacterial performance evaluation.

Introduction

The present doctoral thesis is based on current challenges encountered in clinical setting, specifically in orthopedics. The present work consists of two main chapters that describe the theoretical background on which this thesis has been designed, together with methods and concepts and the experimental part that aims to design and developed nanostructured coatings that can overcome the current drawbacks of the currently available therapies.

Surgical site infections are one of the biggest concerns in medical environment, together with antibiotic resistance which is a major risk for healthcare systems worldwide. Many medical specialties are impacted by the appearance of SSIs and the high antibiotic resistance that make them very hard to treat.

Bone tissue-related disorders and trauma are very frequent among patients worldwide, and most of the available treatments imply the need for surgery. Therefore, the risk of SSIs' appearance is high in this field, and the availability of efficient alternative is a must, especially in cases where implants or prostheses are needed.

The goal in the orthopedic industry is to obtain a product that can minimize the risk of infections and direct the cellular pathways to obtain proper osteointegration and osteoconduction. A proper approach in this regard is to control surface-material interactions.

In order to achieve these goals, this research aims to design and develop bioactive coatings that possess the needed mechanical and biological characteristics.

The following sections of this summary will briefly present the content of this doctoral thesis.

A. Presentation of the research theme: theoretical background, methods and concepts

I. Theoretical background

1. Biomaterials. Nanobiomaterials.

Traditionally, any biomaterial is defined as a material engineered to be used in a medical setting with multiple puroposes such as: to treat, restore or recover the function of a tissue without generating an adverse response from the host. Biomaterials can be grouped as natural, synthetic, or composites and their development involve the interaction between multiple disciplines: chemistry, biology, materials engineering, physics, and computational chemistry [1, 2].

Nowadays, metallic materials [3], ceramics [4], polymers [5], bioglass [6], and composite materials [7, 8] are used as key components in the production of advanced biomaterials, many of them being used in clinical practice on a daily basis [9, 10]. All these materials can be classified depending on the response triggered in contact with the host. Therefore, biomaterials are bioinert (which will cause the formation of a fibrous capsule when interacting with the physiological environment), bioactive (which interacts with the surrounding tissues), and biodegradable (it degrades in a specific timeframe) [11, 12].

An ideal biomaterial should possess the required characteristics for the application for which it is used. Some critical factors that should be considered while selecting a biomaterial are biocompatibility, mechanical properties, cytotoxicity, the release of by-products during the degradation process, etc. Even if many biomaterials have been intensively studied and used in day-to-day practice, nanotechnology has proved that it might be a smarter solution for bioengineered products in the biomedical field [1].

Nanotechnology is one of the main areas of research and has been intensively exploited to discover its potential in all industries. Currently, nanotechnology can be explained as an interdisciplinary field that aims to combine concepts from distinct fields (including, but not limited to: biology, engineering, physics, chemistry, etc.) to design, create, characterize and manufacture materials or various systems in the range of 1-100 nm [1, 13].

Nanomedicine represents an intersecting domain between nanotechnology and medicine and tries to bring benefits to the prevention or treatment of human disease by developing novel nanobiomaterials, diagnostic options, contrast reagents, nanodevices or nanotherapeutics (Figure 1) [14, 15].

A nanobiomaterial can be labeled as any material synthetized in the nano-scale regime (1-100 nm) which is designed to be used in biomedical applications [1, 17].

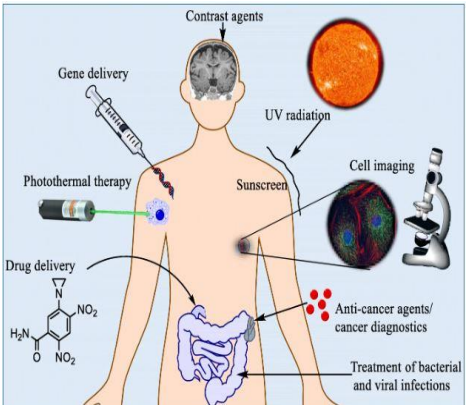

Figure 1. Selection of most common applications of nanotechnology in medicine. Reprinted from an open-access source [16].

Specifically, nanobiomaterials can provide extra features to a conventional biomaterial due to the size-dependent effect. For example, a nanocomposite made of collagen and calcium phosphate replicates the nanostructure of the native bone architecture – natural systems have nanoscale hierarchical compounds, therefore being more efficient than a bulk biomaterial [18]. The investigation of nanobiomaterials has been a point of interest in the past decades in many areas of the biomedical field [19-21].

2. Bone tissue – the basics

In accordance with recent estimations for bone fractures, the incidence is continuously growing, especially in patients that are affected by genetic diseases, age-related disorders, and bone tissue illnesses (e.g., osteoporosis, arthritis). In 2020, a statement was made by the International Osteoporosis Foundation according to which at every 3 seconds, osteoporosis causes a fracture, summing up about 9 million per year globally. The normal healing process (bone remodeling) in native bone is the most outstanding regeneration process because it results not in scar tissue but in a genuine reconstruction of the native structure. However, the healing failure rate is up to 10% in these patients due to the restrictive self-healing ability of the affected tissue. Currently, the clinically available treatments rely on medication such as biophosphonates, raloxifene, or teriparatide, which intends to reduce the risk of fractures, but the regenerative process is still slow and complicated [22, 23].

Natural bone represents a highly dynamic structure formed by mineralized connective tissue that involves the action of four key cell types: osteoblast, osteoclasts, bone lining cells, and osteocytes. The bone remodeling process involves the synchronized activity of these cells, based on the ability of osteoclasts and osteoblasts to simultaneously resorb and form new bone [24].

2.1. Materials of choice in bone tissue applications

2.1.1. Hydroxyapatite

The composition of bones consists of collagen type-I, mineral phase (about 65-70%), lipids, non-collagenous proteins, and other molecules. The architecture and interaction among these components provide tissue properties [25]. It is well known that the calcium phosphate family is a category of biomaterials commonly used for biomedical applications, particularly in orthopedics and dentistry [26]. Certain attention has been given to hydroxyapatite (HAP), chemically expressed as $Ca_{10}(PO_4)_6(OH)_2$, given the fact that it is very similar in structure to biological apatite. HAP can be isolated from natural sources (fish bones, shrimp shells, eggshells, etc.) – natural HAP, or synthetic – which can be synthetized by various techniques [27].

A lot of studies have been published over the past years that prove the potential of hydroxyapatite for use in diverse biomedical applications [28-31]. Yet, with the benefit of nanotechnology, many of its properties have been improved, including antimicrobial activity known to be poor in ceramics. Nonetheless, its nanostructure offers the advantage of a high crystallinity that permits ion substitution, being a real benefit that allows it to expand its uses for biomedical purposes [31].

2.1.2. Magnesium phosphate

The first use of Mg alloys in biomedical field was more than 2 centuries ago, but the potential of magnesium-based materials was intensively studied in the last decades due to major expansion in processing and manufacturing practices [32]. Starting from this point, many studies have been conducted to investigate Mg alloys *in-vitro*, specifically its impact on human mesenchymal stem cells (MSCs), mouse fibroblasts, bone-derived cells, and MG-63 human osteosarcoma cells. *In-vivo* studies were numerous as well, involving studies on rat femora, guinea pig femora, implantation of rods into rabbit tibiae, etc. [33].

3. Antibacterial approach in the biomedical field

3.1. Biofilm formation

It is well known by now that *Gram-positive* and *Gram-negative* bacteria are the principal protagonists in SSI development, most of the SSIs being caused by the staphylococcal class (*Gram-positive* bacteria). The main differences between these bacterial types are presented in Figure 2 [34, 35].

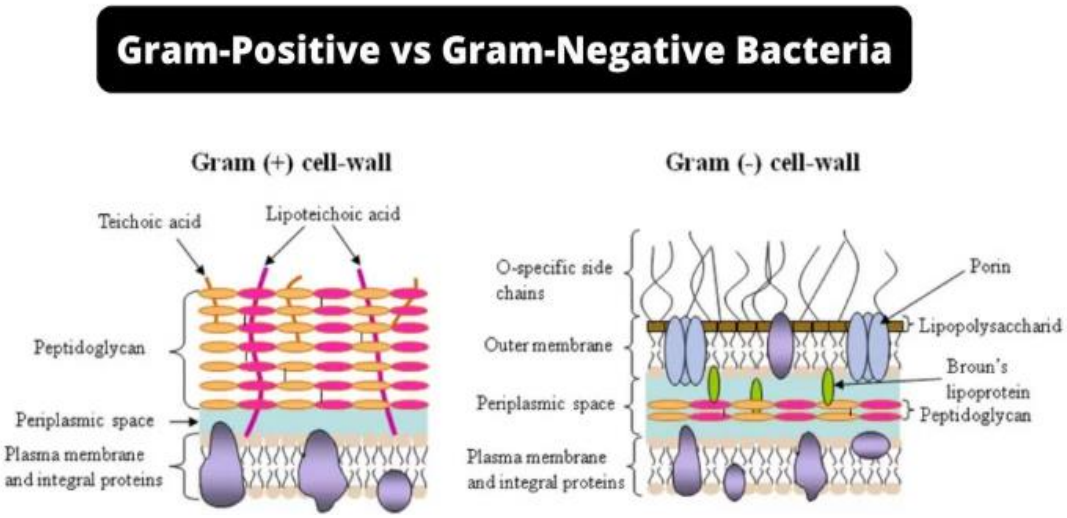

Figure 2. The most important differences in the structure of the two types of bacteria. Illustration redistributed from an open-access source [34].

Surgical infection often occurs from bacteria originating from instruments or hands of the hospital staff during the intervention, from patient's skin, contaminated products, or other distant local infections [35].

The appearance of SSIs is strictly dependent on the biofilm formation process. This is a successive process that occurs in stages, starting with bacterial adhesion on a specific surface (e.g., implant, prosthesis, etc.) (stage 1). The adhesion process that occurs in the first stage is reversible. At this point, numerous factors are accountable for the initial adhesion: London van-der-Waals forces, Brownian movement or polarity, leading to microcolony materialization. However, bacteria must be in the proximity of the implanted surface for successful adherence. From this stage, microcolonies are formed by aggregation and are bounded in an extracellular polysaccharide matrix (EPM), where they turn out into a macro-colony, so the adherence process becomes

irreversible [36 - 38]. The EPM and secreted extracellular polysaccharide substances (EPS) possess an important function in building the architecture of the biofilm, representing the 2nd stage – growth and maturation. Additionally, non-adhered cells escape from the matrix. Therefore, they are able to drift to other sites to go over the same process. This represents infection spreading and is the 3rd and final stage of biofilm formation [35].

It is well known by now that the inaccurate prescription and administration of antibiotics have represented one of the biggest concerns in terms of resistance. There are several mechanisms of drug resistance described at this point. The main types are known in the literature as targeted modification by enzymatic changes, targeted substitution, antibiotic destruction or modification, targeted modification by mutation, restricted permeability of antibiotics, etc., all of them serving the same purpose: to block antibiotic binding to its specific target. Therefore, biofilm activator pathogens are extremely hard to treat by conventional means, so novel tactics need to be developed [39].

3.2. Nanotechnology in preventing biofilm formation

Development in the nanomaterials field has led to the hypothesis that they might be good candidates for inhibiting pathogenic activity in the host with minimal side effects. Various nanoparticles have been generated by diverse synthesis techniques to overcome conventional methods' existing drawbacks. Nanoparticles such as copper, gold, magnesium, and silver on their own or in combination with other compounds, showed promising results in bacterial inhibition [40].

3.3. Silver nanoparticles – AgNPs

Antibacterial efficiency of silver salts is well-known since ancient times, and this property has been exploited in different industries ever since [41, 42]. This effect strictly relies on their physico-chemical characteristics like surface, shape or size. Research suggested that the smallest particles in the nanometer range promote cellular death by improving the permeability of silver ions within the microbial specimens [43].

3.4. Zinc oxide nanoparticles

Zinc oxide (ZnO) can be categorized as multifunctional as a result of its outstanding physical and chemical properties. It can be defined as a semiconductor (II-IV group) with superior chemical, mechanical and thermal stability, a broad range of radiation absorption, and high bond energy, characteristics that lead to its use in various industries including, but not limited to: electronics, laser technology, sensors (due to its pyro- and piezoelectric features), energy generation, etc. Due to its biocompatibility, strong UV absorption, good degradability rate, and low toxicity, the interest in using ZnO in cosmetic and biomedical applications has risen substantially in the last years [44].

Nanostructured ZnO proved to possess superior properties due to morphology and dimension of the particles. Though, their behavior in the physiological environment is dependent on surface chemistry, particle morphology, size, or reactivity in solution. As a result, the synthesis method should provide ZnO nanostructures with controlled sizes, uniformity, and proper morphology [45].

3.5. Gold nanoparticles

Usually, AuNPs with small diameters (max hundreds of nm) are established in organic solvents or aqueous solutions. Typically, $AuCl_3$ is reduced by a specific substance which provokes the nucleation of Au ions into nanoparticles. Additionally, a surfactant is added with the role of a stabilizing agent. The surfactant is chemically bounded to the AuNPs surface and is charged to induce the repelling effect between particles, promoting colloidal stability. Recently, gold nanostructures and their properties have headed to new approaches with huge potential in biomedicine and clinical applications [46, 47].

In biomedical applications, AuNPs are a good choice for an extensive range of biomedical applications due to enhanced biocompatibility, good chemical stability and facile bioconjugation with multiple types of molecules. Moreover, the availability of numerous synthesis methods represents another big advantage. Gold nanoparticles can be obtained by using bottom-up or top-down approaches. The bottom-up approach includes methods such as chemical/thermal reduction. Top-down methods embrace techniques like electron beam lithography or photolithography etc. Reduction methods refer to building atoms into the preferred structure by ion reduction process, while lithography methods involve the removal of matter from the raw (bulk) material in order to

obtain the desired structure in nanoscale range. Even if both approaches are suitable for getting the desired shapes or structures, they present some limitations: bottom-up techniques may be subjected to poor mono-dispersity, whereas top-down methods may produce extensive material loss. However, the latest development in this area led to the expansion of green synthesis practices by using biomolecules, microorganisms, or plant-based constituents [48].

3.6. Copper nanoparticles

Copper nanoparticles (CuNPs) have multiple applications in numerous industries, including, but not limited to: optics, medicine, conductive coatings, biomedical, lubricants, dental, cosmetics, or nanofluids. Additionally, it is well known that copper complexes (salts) have been a good choice in terms of disinfectants due to their antiviral and antibiotic performance. Studies suggested that their antimicrobial behavior is improved at nanoscale level compared to classic cooper salts due to their crystallographic surface structure [49-51].

4. Surface modification

The first interaction between any biomaterial and the host starts at the surface level. Surface features such as morphology, elasticity, charge, topography, or wettability are fundamental parameters when biological fluids encounter a foreign device. Specifically, the cellular differentiation pathway of stem cells can be induced and controlled by morphology and topography as well as the proliferation rate, while hydrophilic surfaces attract cell adhesion [52].

Surface modification is one of the most common and complex strategies to overcome multiple issues (low integration, implant rejection, site infection, etc.). Numerous tactics have been elaborated over the past decades, being classified as passive or active approaches. In terms of passive strategies, the biocidal effect can be achieved by customizing physicochemical features like topography, surface wettability, chemistry, etc. [53].

One of the most common active tactics, currently used in clinical settings is represented by antibiotic-release coatings. The idea is to immobilize the antibiotic on a layer with a controlled degradation rate or to integrate the substance within a coating that performs as a reservoir. In both situations, the antibiotic release should be controlled and should take place over a specific

timeframe [53]. The advantage of these methods is that the mechanisms by which the release takes place offer the possibility to deliver high amounts of active substances locally. Such mechanisms include hydrolysis, biodegradation, or diffusion. However, there are some limitations to this treatment like the accumulation of proteins, which may conduct to the insufficient release of the active substance or inadequate reservoir capacity. An alternative to these coatings is known as contact-killing surfaces. The concept is based on anti-adhesive molecule immobilization on the surface by covalent bonding to repel bacteria attachment [52].

4.1. Surface features in antimicrobial coating design

The interactions that take place in the earliest stages of contact are electrostatic forces that guide cellular and bacterial attachment. According to zeta-potential measurements, most of the bacteria species possess a negative net charge, therefore, they attach tight and fast to substrates that have a positive charge. During the initial stages of approach and adhesion, bacteria encounter short-range repulsions when they get closer to negatively charged surfaces [54].

One of the most influential surface parameters that can change the surface energy of the material and can inhibit the initial adhesion of bacterial strains is represented by nanoscale topography. At this level, topography can change the surface's physical and chemical properties, including surface energy. It was well demonstrated over time that topographical features like roughness, chemistry, or surface geometry are able to influence bacterial adhesion. Recently, researchers focused their resources on designing surface topographies that can kill bacteria and inhibit their attachment [55, 56].

Surface wettability can influence many processes related to bacterial infections, including adhesion, friction, adsorption, or lubrication. A surface classified as moderately wettable can induce bacterial attachment due to hydrophobic interactions and hydrogen bonding [55].

4.2. Surface modification techniques

4.2.1. Matrix-assisted pulsed evaporation method

An advanced and promising surface modification technique that has gained a lot of interest in recent decades is known as matrix-assisted pulsed laser evaporation, or, shortly, MAPLE [57].

A major benefit of this fast and versatile technique is the possibility of building up multi-layered structures when compared to other conventional tactics. Since its earliest stages of development (in 1990), MAPLE was used in the building a large spectrum of structures with different materials, including, but not limited to thin films composed of proteins, polymers, bacteria, living cells, nanostructured layers, etc. The base principle of this technique is that it offers the possibility to gently transfer molecules that vary in molecular weight from the condensed to vapor phase. The material is converted into a frozen target by dilution into a non-interacting solvent and, second, by a frozen process at liquid nitrogen temperature. The newly frozen target is exposed to a pulsed laser beam. At this stage, the conversion into thermal energy takes place due to the energy absorption process. Subsequently, the solvent is vaporized, and the vacuum system allows its evacuation. Basically, when the pulsed laser hits the frozen target, 2 photo-thermal processes are initiated: frozen target evaporation and collection of the released material on the substrate–thin film formation [57, 58]. A simplified scheme of the MAPLE technique is presented in Figure 3.

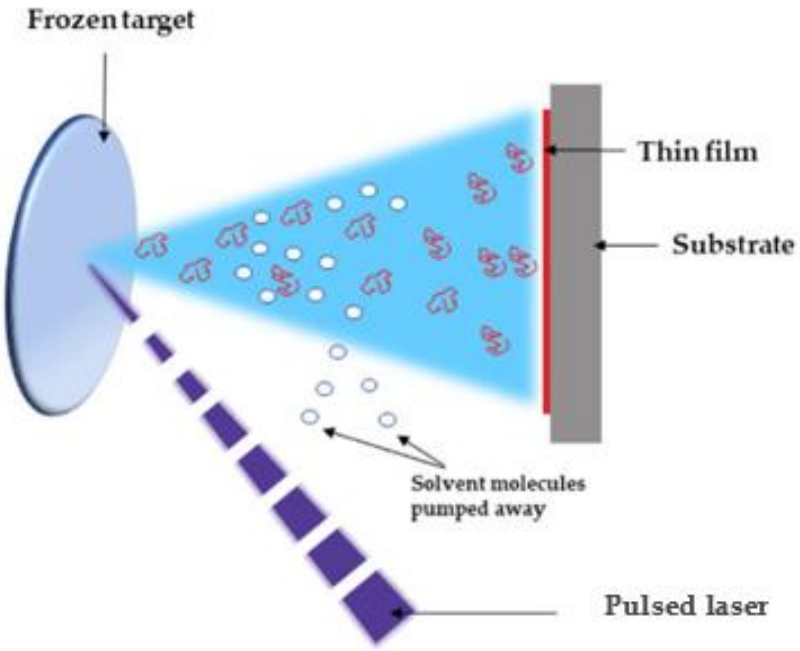

Figure 3. Simple representation of MAPLE deposition technique. Reprinted from an open-access source [59].

Since it can handle nanostructures very precisely and allows uniform biomimetic film deposition, this novel technology has been investigated in surface modification field for biomedical purposes as well. Lately, multiple strategies have been inspected, especially for the production of antibacterial surfaces. For instance, nanofilms based on magnetite core-porous SiO_2 structures, which were functionalized with eugenol, were deposited by MAPLE technique as a potential candidate for antibacterial and biocompatible coatings. The efficiency of the deposition was confirmed by physico-chemical assessment. In-vitro investigations showed a remarkable inhibition in bacterial attachment [139]. Another approach was to synthesize PLGA – poly (lactic-co-glycolic acid) microspheres embedded with magnetite nanoparticles which were subjected to lincomycin functionalization and deposited by MAPLE technique so as to obtain highly biocompatible and antimicrobial coatings. The successful deposition was achieved, and the substrates proved effective against *S. aureus* after in-vitro evaluation [60].

5. Research context

The potential of nanotechnology is being exploited in the medical field for decades now. Many types of nanostructures have been confirmed as proper alternatives to limit infections and overcome the antibiotic resistance issue. As described in the previous sections, AgNPs are nanoparticles of choice in this regard, thanks to their well-known properties and long-term use in antibacterial applications in various industries.

The goal in the orthopedic industry is to obtain a product that can minimize the risk of infections and direct the cellular pathways to obtain proper osteointegration and osteoconduction. A proper approach in this regard is to control surface-material interactions.

In order to achieve these goals, this research aims to design and develop bioactive coatings that possess the needed mechanical and biological characteristics.

In summary, two directions of research were followed based on the same surface modification technique: MAPLE deposition. The first approach was represented by creating bioactive nanostructured coatings consisting of magnesium phosphate ($Mg_3(PO_4)_2$) and silver nanoparticles. This combination of materials was chosen in order to use the antimicrobial properties of AgNPs together with the ability to stimulate bone regeneration provided by $Mg_3(PO_4)_2$. Regarding the synthesis methods, AgNPs were achieved by chemical reduction method and $Mg_3(PO_4)_2$ was obtained by cooling bath reaction. Both synthesis methods proved to be highly efficient. In terms of coating deposition, MAPLE method led to a homogenous deposition of the coating on the substrate, with a controlled size, thickness, and rugosity.

Evaluation of AgNPs was performed by using multiple characterization techniques: XRD (X-Ray Diffraction), TEM (Transmission Electron Microscopy) and SAED (Selected Area Electron Diffraction) which proved a successful synthesis as shown in Figure 4. In a similar manner, characterization of $Mg_3(PO_4)_2$ was performed as showed in Figure 5. All the obtained results led to the conclusion that both compounds were successfully synthetized through the above-mentioned methods.

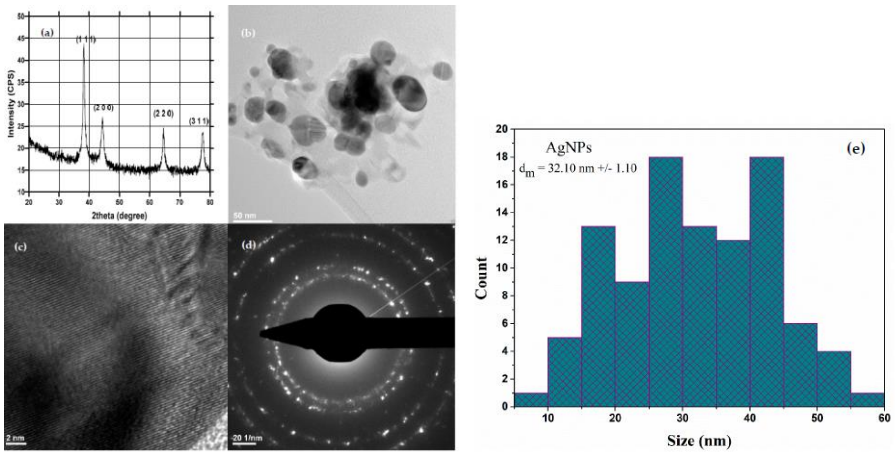

Figure 4. XRD spectrum (a), TEM and HR-TEM micrographs (b,c), SAED patterns (d) and size distribution (e) of AgNPs.

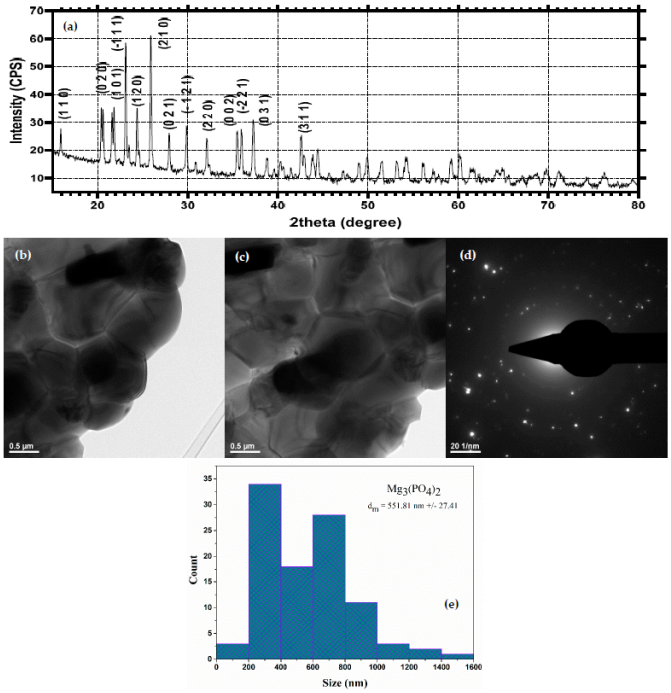

Figure 5. XRD spectrum (**a**), TEM micrographs (**b**,**c**), SAED patterns (**d**) and size distribution (**e**) of $Mg_3(PO_4)_2$.

The evaluation of AgNPs/$Mg_3(PO_4)_2$ coatings was performed by IR-mapping which were obtained for different laser fluences (Figure 6 and 6'). The conclusion was that the optimal laser fluence was determined at 300 mJ/cm^2 without affecting the functional groups or the chemical structure of the coating.

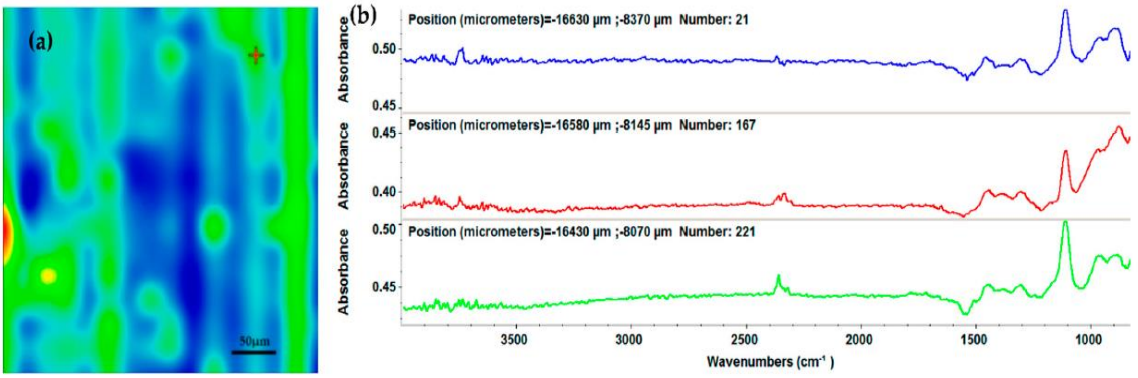

Figure 6. IR map (**a**) and FT-IR spectra (**b**) of AgNPs/$Mg_3(PO_4)_2$ drop-cast.

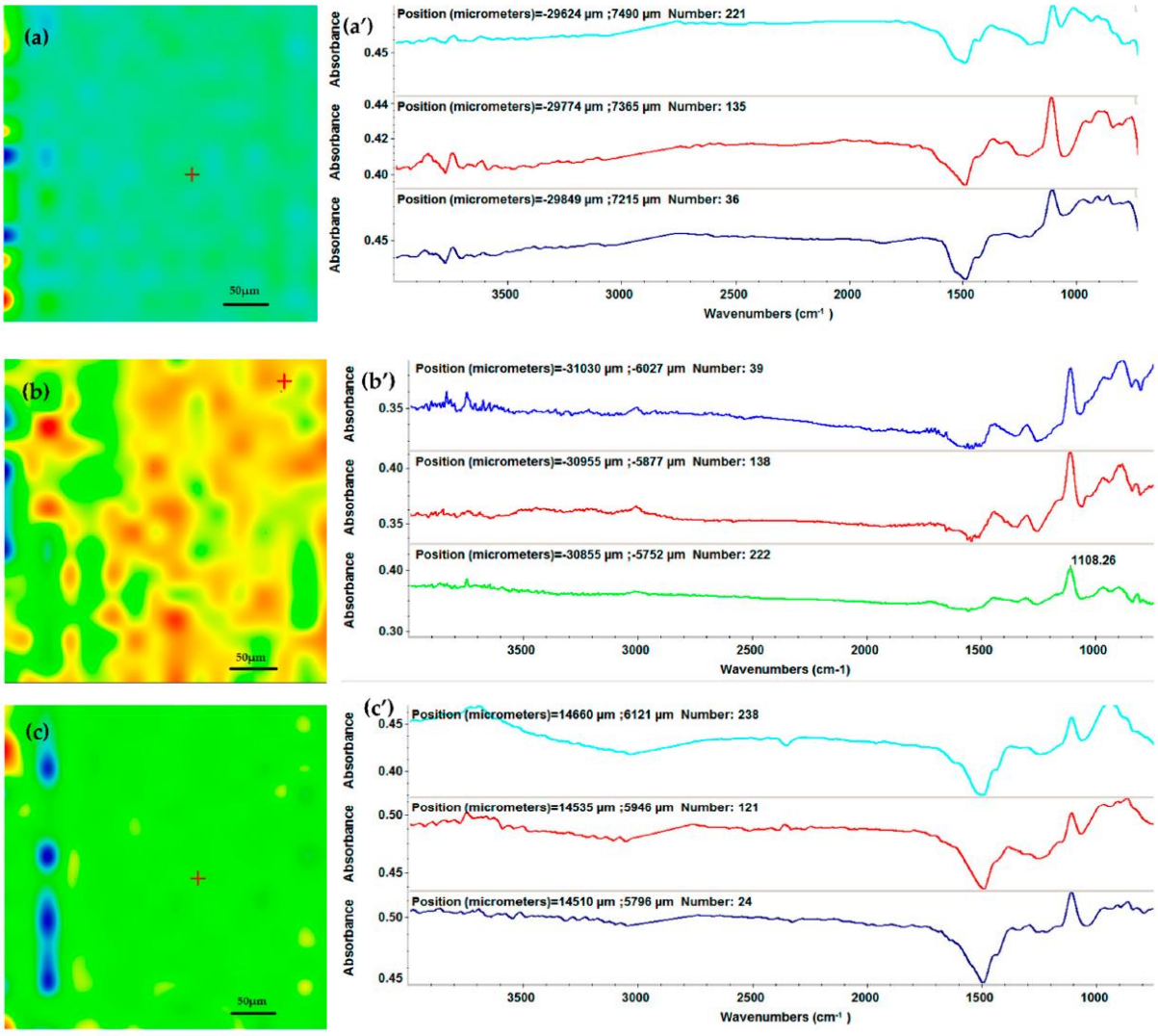

Figure 6'. IR map (**a**) and FT-IR spectra (**b**) of AgNPs/$Mg_3(PO_4)_2$ at 200 mJ/cm^2 (a,a'), 300 mJ/cm^2 (b,b'), and 400 mJ/cm^2 (c,c') laser fluences.

The uniform deposition of the coatings on substrates through MAPLE technique was proved by Scanning Electron Microscopy (SEM images presented in Figure 7. The results obtained by SEM

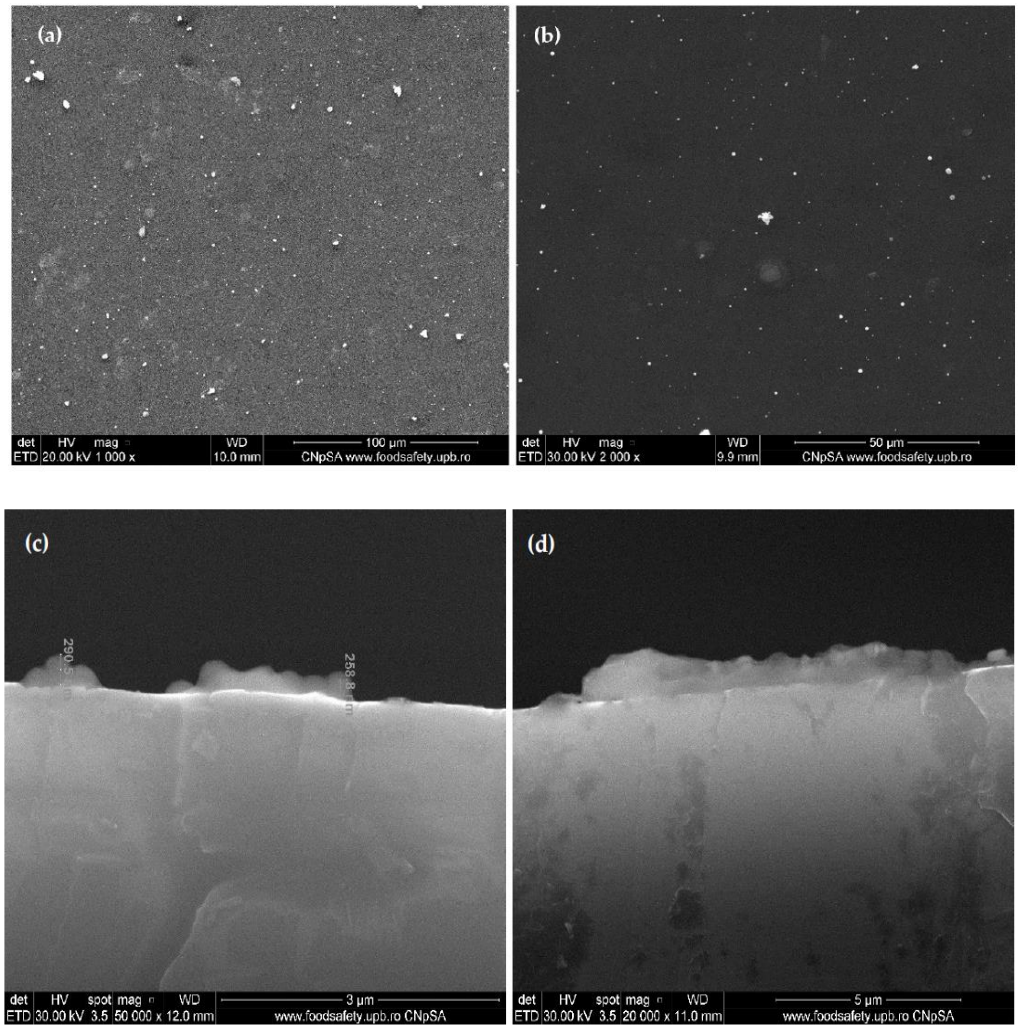

Figure 7. Top-view SEM images (a,b) and Cross-section SEM images (c,d) of the $AgNPs/Mg_3(PO_4)_2$

in cross-section confirm the presence of agglomerates within the coatings with a size range of 55 nm – 300 nm.

In vitro evaluation was assessed by using MC3T3-E1 osteoblasts in contact with the coatings. Cytoskeleton evaluation was performed using fluorescence microscopy (Figure 8) and the results obtained suggested that the morphology and confluency of osteoblasts were

influenced in a positive manner by the addition of the coating on the substrate, presenting well-organized actin filaments and a healthy architecture, proving the efficiency of the coatings.

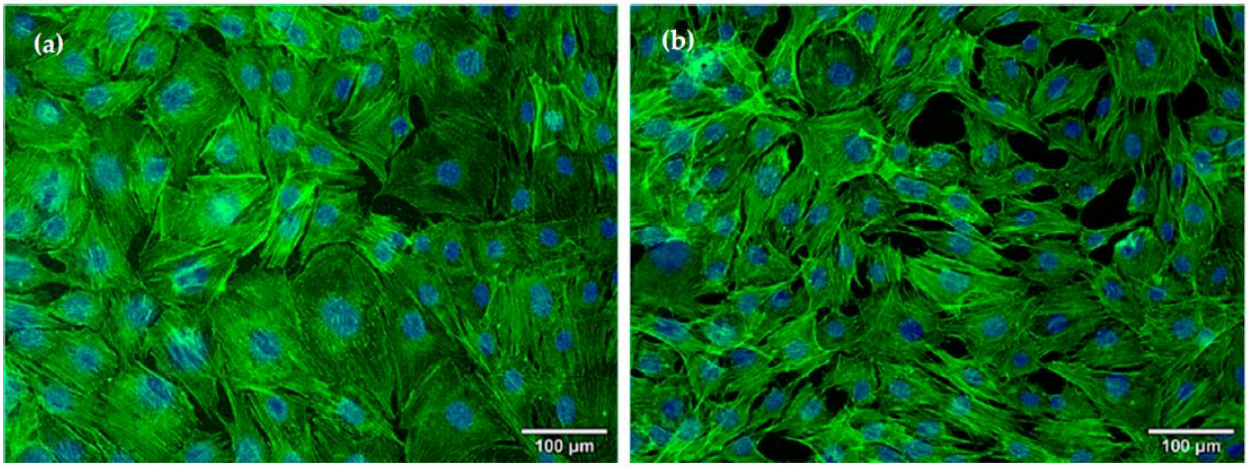

Figure 8. Fluorescence microscopy images for actin filaments (green) and nuclei (blue) staining in osteoblasts grown for 24 h on the surface of control (a), and AgNPs/$Mg_3(PO_4)_2$ coatings (b).

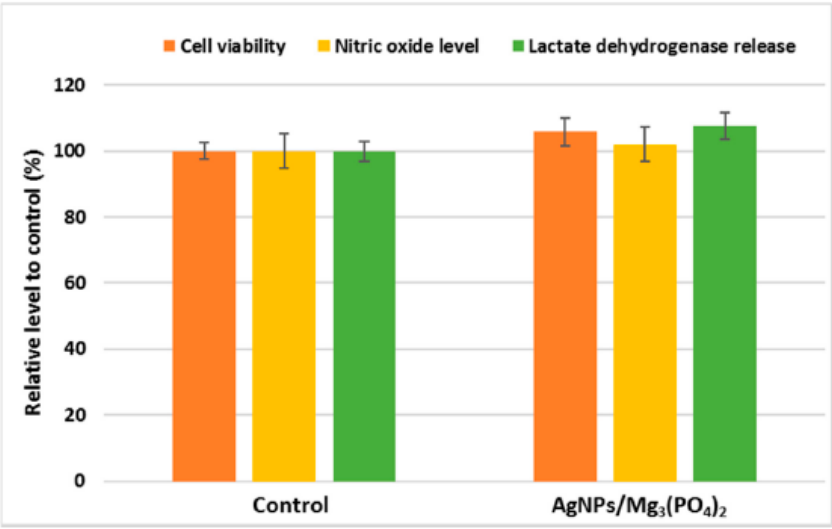

Figure 9. Cell viability, NO level, and LDH release after 24-h growth of MC3T3-E1 osteoblasts on the surface of control and AgNPs/ $Mg_3(PO_4)_2$ coatings.

The MTT assay was used to examine the viability of cells grown on the surface of tested coatings (Figure 9). The obtained results suggested a high survival rate of osteoblasts seeded on the coatings, the levels of cellular viability being higher by 5% compared to control. Furthermore,

no significant changes were recorded for the cells grown on the AgNPs/Mg3(PO4)2 sample, in the case of NO level and LDH release, compared to control.

The models used for assessing the antibacterial performance of the coatings were represented by *S. aureus* and *P.aeruginosa* strains, which were incubated in the presence of the samples for 48h. The measurements were taken at two different time moments: after 24h and 48h after incubation (Figure 10). The results suggested a strong bacterial inhibition for the samples were compared to control.

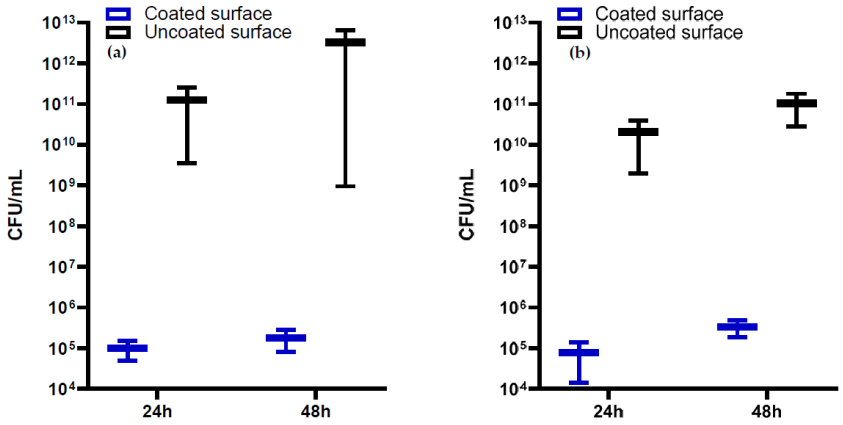

Figure 10. Antibacterial efficiency assessment of the $AgNPs/Mg_3(PO_4)_2$ coatings against S. aureus (a) and P. aeruginosa (b) at 24 and 48 h.

In conclusion, the present study aimed to develop a series of biocompatible and antibacterial coatings that can be used in orthopedic applications. The investigated materials consisted of AgNPs and $Mg_3(PO_4)_2$, which were synthesized through well-known routes and combined for obtaining nanostructured coatings by the MAPLE technique. The study demonstrated the efficiency of the $AgNPs/Mg_3(PO_4)_2$ coatings in promoting osteoblast viability and proliferation while exhibiting antibacterial activity against two of the most important etiological agents of orthopedic implants associated infections. Specifically, the potential of the proposed materials resides in the synergistic effects of $Mg_3(PO_4)_2$ to ensure bone regeneration and implant integration, while AgNPs provide the antibacterial properties of the composite materials.

On the other hand, the second approach was represented by the development of hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ and $Mg_3(PO_4)_2$ thin bioactive coatings that contained BMP4 (bone morphogenetic protein) growth factor for fast osteointegration and were further nebulized with CXF (ceftriaxone antibiotic) for peri-implant infection prevention. Physico-chemical characterization of the samples was performed by using the following techniques: SEM, TEM, SAED and Infrared Microscopy (Figure 11). The results obtained confirmed the success of the synthesis routes.

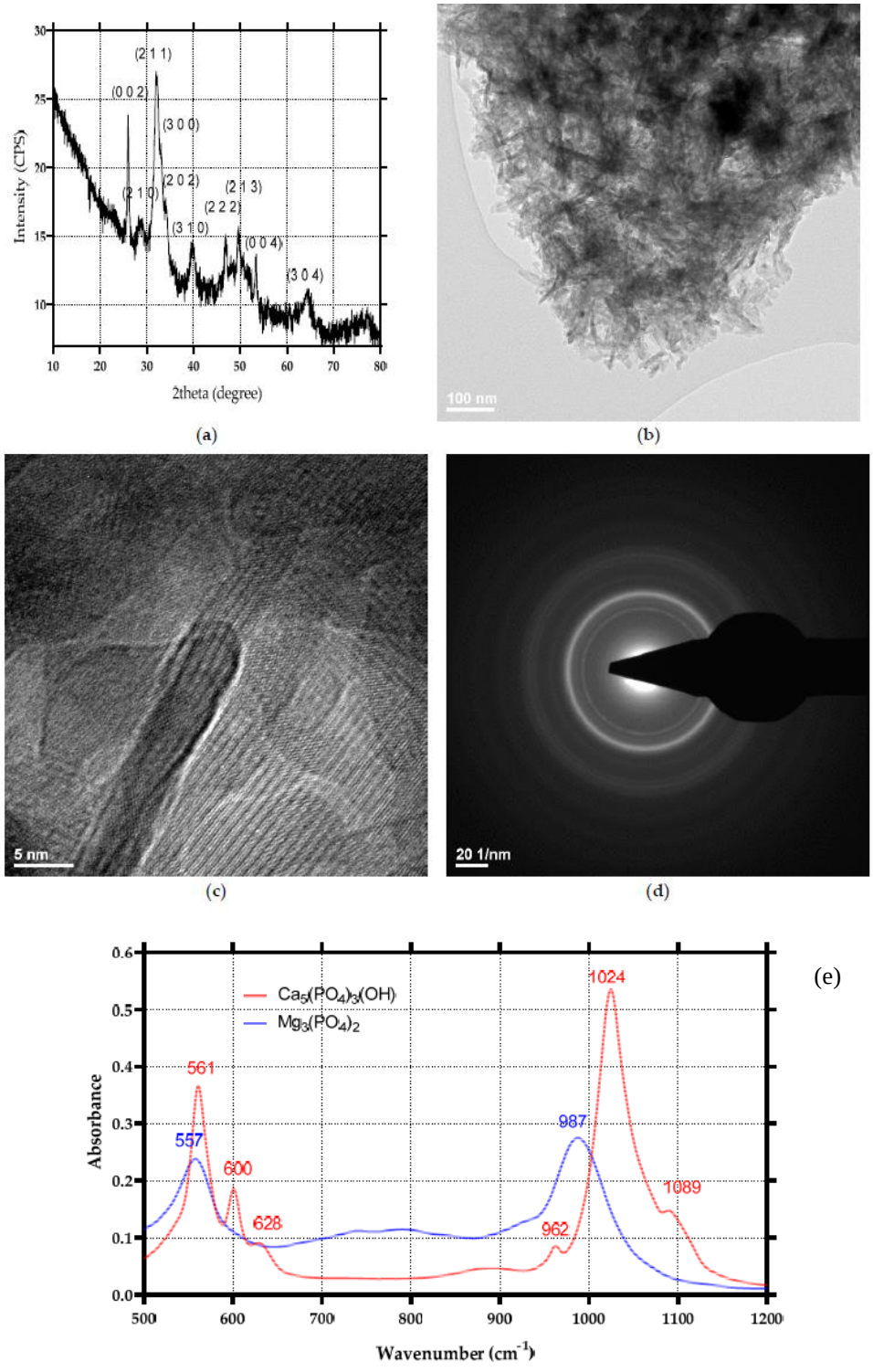

Figure 11. X-ray diffractogram (**a**), TEM image (**b**), HR-TEM image (**c**), SAED pattern (**d**) for HAP nanopowders and IR spectra for HAP and $Mg_3(PO_4)_2$ nanopowders (**e**).

The integrity of the coatings deposited by MAPLE were assessed by IR mapping and IR spectra in order to determine the status of the functional groups. In the same manner as for the previous experiment, three laser fluences were used and according to the results provided by IR analysis, samples deposited at a laser fluence of 400 mJ/cm^2 were chosen for further investigation and nebulization with CXF.

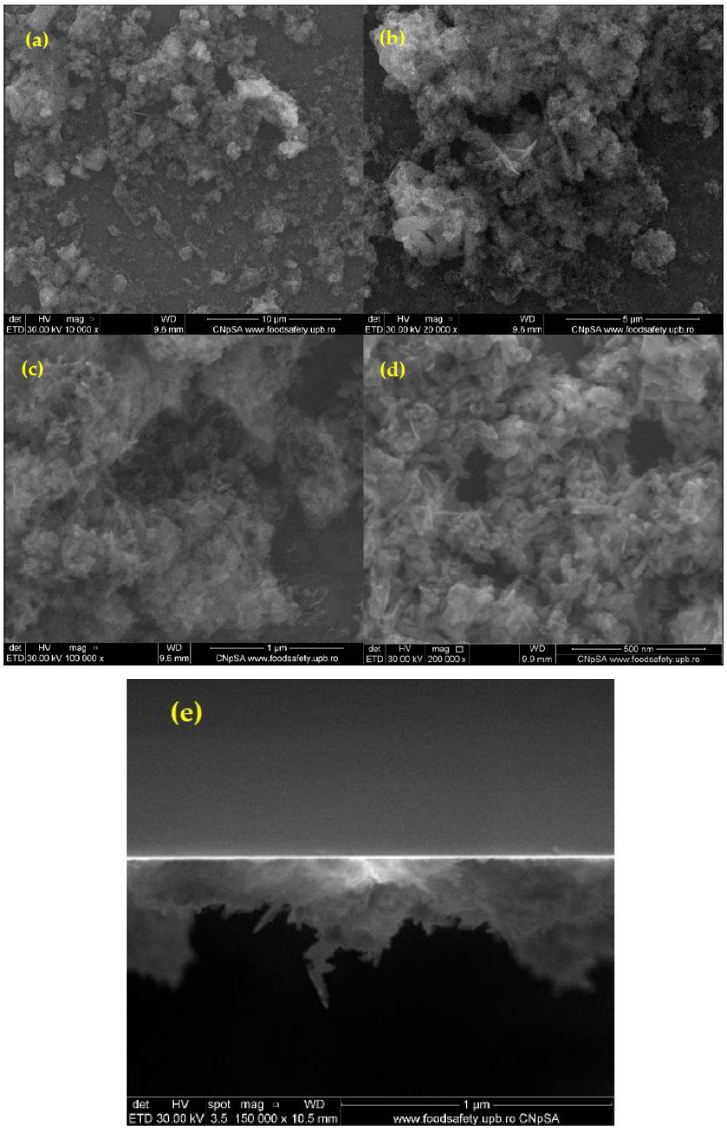

Figure 12. SEM images at various magnifications (a—10,000x, b—20,000x, c—100,000x, d—200,000x) and cross-section (e) for HAP/ $Mg_3(PO_4)_2$ /BMP4 coatings.

Furthermore, the HAP/ $Mg_3(PO_4)_2$/BMP4 coatings were subjected to SEM analysis to investigate the surface morphology of the prepared coating (Figure 12). In this context, the SEM images reveal a relative distribution of the coating on the substrate with the presence of both HA and $Mg_3(PO_4)_2$ powders. The coating has several agglomerates, and the thickness of the tested coating varies from 200 to 500 nm (Figure 12e).

In this study, the bioactive coatings were further studied for their cytocompatibility and antimicrobial properties. Figure 13 depicts the fluorescence microscopy images of the cells grown on the HAP/ $Mg_3(PO_4)_2$/BMP4 coatings labeled with phalloidin-FITC. It can be stated that the HAP/ $Mg_3(PO_4)_2$/BMP4 coatings did not induce any morphological changes in MC3TE-E1 osteoblasts, which exhibited normal shapes suggesting a healthy status. The osteoblast-specific architecture and high monolayer confluency could be observed on the surface of coatings, with central nuclei and elongated actin filaments.

Moreover, the MTT assay was performed for the HAP/ $Mg_3(PO_4)_2$/BMP4 coatings to evaluate the effect of BMP4 in terms of cellular viability (Figure 14). The percentage of live cells grown on the nanocoated surface was in the proximity of control values after 24 h and 72 h, demonstrating the release in the active form of the BMP4, which has been able to facilitate the cell attachment and maintain a normal osteoblast proliferation. The lack of cytotoxicity towards the MC3T3-E1 cells was also confirmed by the levels of NO and LDH release, which were comparable to those obtained for the control sample after both intervals of incubation.

The results presented in Figure 15 show that the obtained nanocoating is releasing the antibiotic in an active form, and it protects the surface colonization by killing the planktonic cells attached to the implant surface, therefore the antibiofilm performance was successfully confirmed.

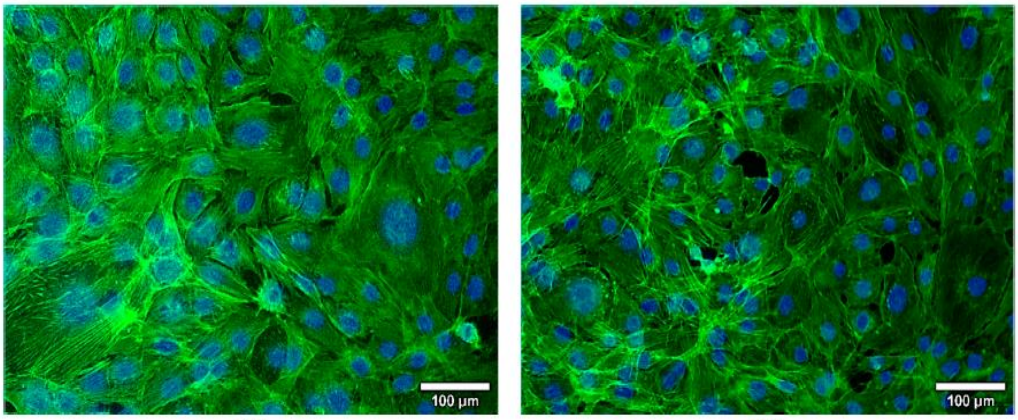

Figure 13. Fluorescence images of actin filaments of MC3T3-E1 osteoblasts cultured at 24 h on the uncoated control (a) and on the composite HAP/ $Mg_3(PO_4)_2$/BMP4 coatings (b).

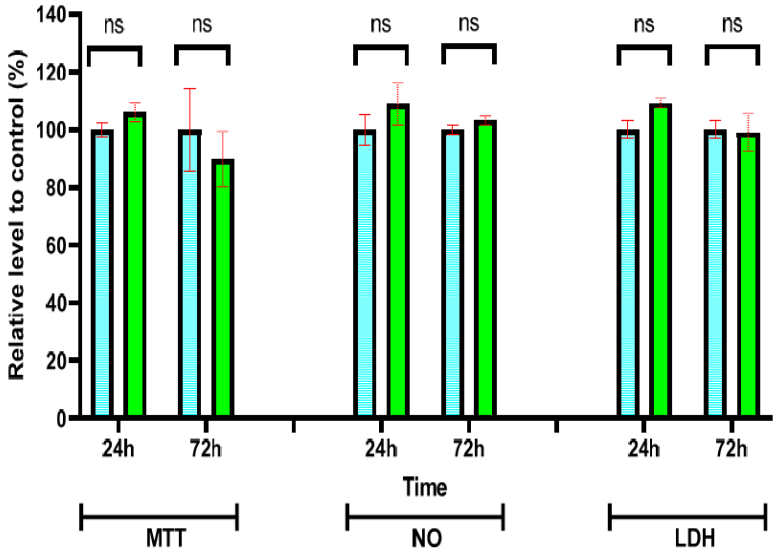

Figure 14. Cellular viability, NO level, and LDH release after 24 h and 72 h of MC3T3-E1 osteoblasts' growth on the control surface and in contact with HAP/ $Mg_3(PO_4)_2$/BMP4 coatings.

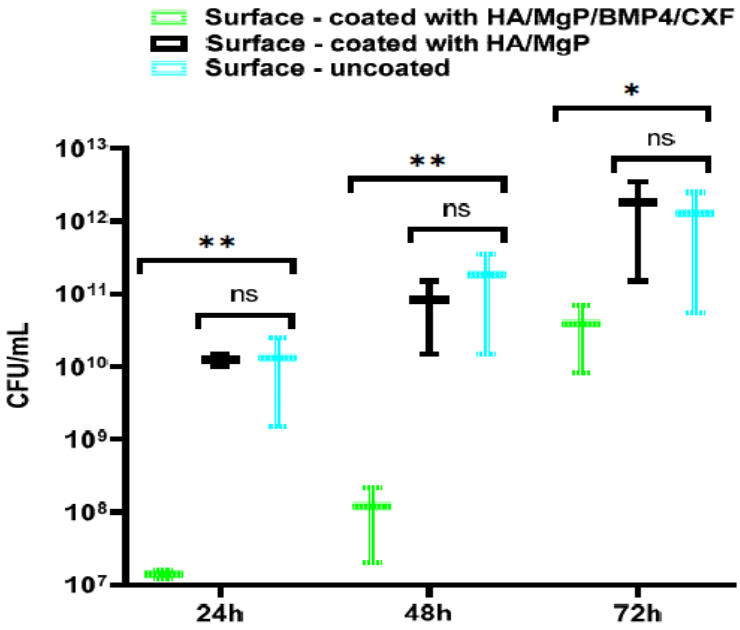

Figure 15. Antibiofilm efficiency assessment of the uncoated and coated surfaces against S. aureus at 24 h, 48h, and 72h.

This study aimed to combine the advantages of two ceramic materials with well-known benefits within bone tissue applications, namely HA and $Mg_3(PO_4)_2$, with those of the BMP4 growth factor and of a large spectrum antibiotic for developing bioactive coatings for bone implants. The experimental study demonstrated the successful synthesis of HA and $Mg_3(PO_4)_2$ powders, followed by optimal material deposition by the MAPLE technique at 400 mJ/cm2 laser fluence. Furthermore, the composite coating proved to exhibit a significant antimicrobial and antibiofilm effect, especially in the first 48 h of colonization, and biocompatibility in relation to osteoblast cells, as revealed by their normal morphology, viability, membrane integrity, and actin cytoskeleton, thus confirming the potential of the present system for developing novel implant coatings.

Conclusions

This thesis begins with a comprehensive literature study, describing the progress made in the orthopedic field in order to find suitable remedies that can overcome the existing drawbacks of clinically available treatments. Nowadays, the use of materials for designing biomimetic alternatives that can exploit the native human body's ability of self-regeneration is a focus point in research.

The literature background that sustains this thesis has been summarized in two review papers that cover two major topics: an up-to-date review of hydroxyapatite particles and how they influence the cellular response and surface modification techniques explored for orthopedic applications.

Published papers supporting the literature background of the present work:

1. **PUBLICATION I** - *Impact Factor 2022: 4.778*: Florea, Denisa Alexandra, *et al*. "Surface modification–A step forward to overcome the current challenges in orthopedic industry and to obtain an improved osseointegration and antimicrobial properties." ***Materials Chemistry and Physics 243*** *(2020): 122579.*

2. **PUBLICATION II** - *Impact Factor 2021: 2.838:* Florea, Denisa Alexandra, Cristina Chircov, and Alexandru Mihai Grumezescu. "Hydroxyapatite particles—directing the cellular activity in bone regeneration processes: an up-to-date review." ***Applied Sciences*** *10.10 (2020): 3483.*

Starting from this point, in the practical section of this thesis, the scope was to develop nanostructured antibacterial coatings that are capable of inhibiting the attachment, spread and growth of bacterial cells on substrates and enhancing bone regeneration processes. Novel technologies were used to create and deposit complex thin films with controlled characteristics for improved performance.

For instance, all the coatings developed during the experimental section of this thesis were deposited by MAPLE technique, which offered the possibility of controlled properties: e.g., precise rugosity or thickness, allowing for thin film deposition and meticulous design.

In the first study (Publication III), a series of coatings composed of magnesium phosphate ($Mg_3(PO_4)_2$) and silver nanoparticles (AgNPs) were fabricated to simultaneously acquire anti-infective behavior and faster bone regeneration by osteoblasts proliferation. $Mg_3(PO_4)_2$ nanoparticles were obtained by cooling bath reaction, while AgNPs by chemical reduction method, as described in Publication III – *'3. Materials and Methods'* section. Samples were subjected to physico-chemical characterization by using the techniques described in Publication III – *'3.3. Characterization methods'*. The biological assessment was made to determine the biocompatibility of the thin film. The anti-infective properties were confirmed by testing the samples against *S. aureus* and *P. aeruginosa* which are two of the most challenging bacteria in implant-associated infections.

The efficiency of the deposition technique was evaluated by FT-IR spectra and IR mapping, the integrity of the functional groups being optimal at a laser fluence of 300 mJ/cm^2.

The conclusions gained from this experiment pointed out the efficiency of AgNPs/$Mg_3(PO_4)_2$ coatings which are able to deliver an outcome that can offer both antibacterial properties - due to the use of AgNPs, and improved regeneration together with implant osseointegration - due to $Mg_3(PO_4)_2$ nanoparticles.

The second study (Publication IV) of the experimental section of this thesis was based on manufacturing thin coatings made of hydroxyapatite and magnesium phosphate, loaded with BMP4 growth factor (bone morphogenetic protein), further nebulized with ceftriaxone antibiotic for antibacterial purposes. The same deposition method was selected (MAPLE) and samples were assessed from physico-chemical perspective by using the following methods: XRD, SEM, TEM, SAED, IRM and FT-IR. The efficiency of ceftriaxone was assessed versus *S. aureus*, while cytocompatibility was determined on MC3T3-E1 osteoblastic cell line.

The benefits of using two ceramic materials together with growth factors and antibiotics and the efficacy of the deposition technique were demonstrated in terms of both antibiofilm performance and biocompatibility. The bacterial inhibition proved to be high in the first 48h of colonization, while the biocompatibility in relation to the cell line used for testing was confirmed by membrane integrity, high viability and actin cytoskeleton.

All of the findings from the experimental section of this thesis can be considered part of the foundation of antibacterial thin coatings development. Such coatings can be considered suitable candidates to prevent infection and enhance bone regeneration processes by using MAPLE deposition technique, which represents a novel and impressive method to achieve the desired coating with well-controlled characteristics.

Published papers of the experimental part of the present work:

1. **PUBLICATION III** - *Impact Factor: 6.208 (2021):* Florea, Denisa Alexandra, et al. "Design, Characterization, and Antibacterial Performance of MAPLE-Deposited Coatings of Magnesium Phosphate-Containing Silver Nanoparticles in Biocompatible Concentrations." ***International Journal of Molecular Sciences*** *23.14 (2022): 7910.*
2. **PUBLICATION IV** - *Impact Factor: 3.748 (2021):* Florea, Denisa Alexandra, et al. "Bioactive Hydroxyapatite-Magnesium Phosphate Coatings Deposited by MAPLE for Preventing Infection and Promoting Orthopedic Implants Osteointegration." ***Materials*** *15.20 (2022): 7337.*

Cumulative Impact Factor = 17.572

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