

THE INNOVATIVE OXIDE-BASED
COATINGS FOR THE APPLICATION IN
MEDICINE: UTILAZTION OF THE
 V_2O_5 AND MgO MULTIFUNCTIONALITY

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Doctoral Dissertation
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Doctoral dissertation

INOVATIVNE PREVLEKE ZA APLIKACIJO NA
PODROČJU MEDICINE: UPORABA VEČNAMENSKIH
MATERIALOV NA OSNOVI V_2O_5 IN MgO

Doktorsko delo

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Abstract

In this study, multifunctional polymer-based composite coatings were processed by an intention to couple either i) **magnesium oxide (MgO) contact-based antibacterial activity with bioactive properties of magnesium ions** (dissolved form of MgO) or ii) **the antibacterial properties of vanadium pentoxide (V_2O_5) via biomimetic myeloperoxidase-like catalytic activity with the bioactivity of low concentrations of vanadate ions** (dissolved form of V_2O_5). I investigated correlations i) of antibacterial activity and ii) mechanisms to control metal-oxide solubility kinetics within polymer-based coatings with the structural properties of processed materials.

The appropriate design of MgO surface is crucial at both, the nano and atomic scale because of its contact based mechanism of antibacterial activity. I confirmed that nano-texturing of the MgO microrods's surface improved the antibacterial activity against *Escherichia coli* (*E. coli*, ATCC 47076). The optimal nano-texturing of the MgO microrods was achieved after calcination at 900°C (MgO 900), making them more antibacterially potent than commercially available MgO nanoparticles (MgO NPs), which could not be explained by differences in their morphology or specific surface area. It was rather a consequence of a lower abundance of low coordinated oxygen atoms at MgO surface, which improved its resistance against hydrolysis and antibacterial activity. With respect to the reactive oxygen species (ROS), I proved that their generation was a side product of i) the MgO hydrolysis or ii) the MgO/bacteria interactions rather than an inherent property of MgO.

The catalytic properties of 1D nanostructured V_2O_5 were related to its structural peculiarities. I confirmed that pure orthorhombic V_2O_5 nanowires exhibited stronger myeloperoxidase-mimicking antibacterial activity than the intercalated ($M = H_2O, K$) $M_xV_2O_5$ nanoribbons. The unique ability of V_2O_5 to exhibit self-maintained (no requirement for H_2O_2 in environment) antibacterial activity, based on its myeloperoxidase-like catalytic activity was discovered and described.

The ability of different polymeric carriers and the metal-oxide particle morphology to modulate *in vitro* stability of the prepared metal-oxide/polymer-matrix composite coatings was assessed. I highlighted the correlation between the strength of the interactions at the metal-oxide/polymer interface and the composite's stability under simulated physiological conditions. The appropriate choice of metal-oxide particle morphology allowed targeting both, the particle agglomeration and metal-oxide solubility control. 1D anisotropic particles exhibited reduced agglomeration compared to the isotropic particles, which improved their processing characteristics. In addition, the application of 1D particles instead of isotropic particles resulted in the annihilation of initial burst release of dissolved metal ions.

The optimal properties were obtained for poly(lactide-*co*-glycolide) (PLGA) polymer matrix because it enabled partially exposed 1D metal-oxide particles at its surface upon exposure to the surrounding media, which was crucial for successful prevention of *S. epidermidis* (NCIMB 8853) colonization on their surface while ensuring the absence of any harmful effects on the red blood cells.

Povzetek

Disertacija opisuje sintezo več-funkcionalnih kompozitnih prevlek na polimerni zasnovi z namenom združevanja i) **kontaktnega mehanizma protibakterijskega delovanja MgO z bioaktivnimi lastnostmi magnezijevih ionov** (produkt raztapljanja MgO) ali ii) **protibakterijskih lastnosti V_2O_5 prek mieloperoksidazi podobne katalitske aktivnosti z bioaktivnostjo nizkih koncentracij vanadatnih ionov** (produkt raztapljanja V_2O_5). Raziskoval sem i) povezave med strukturo kovinskih oksidov in antibakterijskimi lastnostmi in ii) kontrolo raztapljanja kovinskih oksidov znotraj prevlek na polimerni zasnovi.

Zaradi kontaktnega mehanizma delovanja je ustrezno oblikovanje površine MgO ključnega pomena tako na nano kot na atomski ravni. Potrdil sem izboljšanje antibakterijske aktivnosti MgO mikropalčk proti *E. coli* z nano-teksturiranjem njihove površine. Optimalna aktivnost je izmerjena za mikropalčke, ki so bile pripravljene na 900°C (MgO 900). Le-te so pokazale močnejši baktericidni potencial v primerjavi s komercialnimi MgO nanodelci (MgO NPs), kar ni bilo mogoče razložiti z razlikami v morfologiji ali specifični površini prahov. Razlog je bila nižja vsebnost nizko koordiniranih kisikovih atomov na MgO površini, kar je izboljšalo odpornost proti hidrolizi in s tem antibakterijsko aktivnost. Nastanek reaktivnih kisikovih spojin (ROS) je bil stranski produkt i) hidrolize MgO ali ii) interakcij MgO z bakterijami.

Katalitske lastnosti 1D nano-strukturiranega V_2O_5 so bile povezane z njegovo strukturo. Potrdil sem bolj učinkovito mieloperoksidazi-podobno katalitsko delovanje čistih ortorombskih V_2O_5 nanožičk od interkaliranih monoklinskih ($M=H_2O$, K) $M_xV_2O_5$ nanotrakov. Dokazal sem lastnost, ki je značilna le za V_2O_5 , da samovzdržuje antibakterijsko aktivnost brez dodajanja H_2O_2 na osnovi mieloperoksidazi-podobne katalitske aktivnosti.

Raziskal sem sposobnost različnih polimernih nosilcev in morfologije delcev kovinskih oksidov, da modulirajo *in vitro* stabilnost pripravljenih kovinski-oksid/polimerna-matrika kompozitnih prevlek. Poudaril sem korelacijo med jakostjo interakcij na kovinski-oksid/polimer fazni meji in stabilnostjo kompozita pod simuliranimi fiziološkimi pogoji. Izbira morfologije oksidnih delcev je vplivala tako na njihovo aglomeracijo kot na njihovo kontrolo raztapljanja znotraj prevlek. 1D anizotropni delci so bili manj aglomerirani kot izotropni delci, kar je izboljšalo njihove procesibilne lastnosti. Prav tako prepreči uporaba 1D oksidnih delcev namesto izotropnih delcev začetni izbruh raztopljenih kovinskih ionov in tako izboljša stabilnost kompozitnih prevlek pod simuliranimi fiziološkimi pogoji.

Optimalno obnašanje kovinskih oksidov sem dosegel ob impregnaciji znotraj PLGA polimerne matrike. Le-ta je omogočila delno izpostavljenost nano-strukturiranih 1D delcev kovinskih oksidov na svoji površini, kar je bilo bistvenega pomena pri preprečevanju kolonizacije *S. epidermidis* ob zagotavljanju odsotnosti škodljivih učinkov na rdečih krvničkah.

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Abbreviations

AFM	... atomic force microscopy
AN	... annealed
ATR	... attenuated total reflection
BET	... Brunauer-Emmett-Teller
CellM	... cell media
CFU	... colony forming units
CIED	... cardiac implantable electronic device
CV	... crystal violet
DCM	... dichloromethane
DHR	... dihydrorhodamine
DMPO	... 5,5-dimethyl-1-pyrroline N-oxide
DRIFT	... diffusion reflection infrared technique
EDS	... electron dispersive spectrometry
EPR	... electron paramagnetic resonance
FBS	... fetal bovine serum
FT-IR	... fourier transform infrared spectroscopy
HR-TEM	... high resolution transmission electron microscopy
HTT	... hydrothermal treatment
LB	... Luria-Brentani
MBC	... minimal bactericidal concentration
MDT	... microdilution test
MH	... Mueller-Hinton
MPs	... microparticles
MS	... mass spectrometry
NPs	... nanoparticles
NWs	... nanowires
PAR	... 4-(2-pyridylazo)-resorcinol
PBS	... phosphate buffered solution
PCL	... polycaprolactone
PGA	... polyglycolic acid
PI	... polydispersity index
PL	... photoluminescence
PLA	... polylactic acid
PLGA	... poly(lactic-co-glycolic)acid
PS	... polystyrene
RBC	... red blood cells
ROS	... reactive oxygen species
SAED	... selected area electron diffraction
SEM	... scanning electron microscopy
TEM	... transmission electron microscopy

TGA	... thermogravimetric analysis
THR	... total hip replacement
TKR	... total knee replacement
UV	... ultraviolet
XRD	... X-ray diffraction

Chapter 1

Introduction

1.1 Biomaterials in Medicine

Implanted biomedical devices (orthopaedic implants, artificial joints, vascular grafts, blood vessels, pacemakers, intraocular lenses etc.) are frequently applied for resolving various health problems and improving the quality of life, especially in the elderly population [1]. Apart from the long-term implants, various temporary devices are also used as adjuncts to most medical procedures [2].

After Second World War, the surgeons were dealing with the increasing number of patients requiring the replacement of either damaged or diseased body parts. At the same time, the high-performance metal, polymer and ceramic materials transitioned from restricted (during the war) to available (after the war in peacetime) status. As a result, materials originally manufactured for automotive industry were taken “off the shelf” by surgeons and applied as biomaterials for replacement of the dysfunctional body parts. They included silicones, polyurethanes, Teflon, nylon, methacrylates, titanium and stainless steel [3]. The experience with a range of initially-applied materials used as biomaterials allowed scientists and surgeons to gain a valuable knowledge about the necessary requirements for implanted biomaterials to perform their intended *in vivo* functions. The ability of implanted biomaterial to perform the function inside the organism is dependent on many factors like their composition and mechanical properties, surface topography and molecular landscape, among others. However, the long term success or failure of a biomaterial implantation procedure depends on the ability to resist infection and resolve the host response [4].

The progress in the polymer science as well as broadening of the understanding of the biological cascade of events which take place after implantation of a biomaterial are nowadays providing the foundation for the development of field of tissue engineering. Current state of the art includes processing of biodegradable biomimetic biomaterials in the form of functionalized biodegradable scaffolds. At the beginning of the recovery process, they should provide mechanical stabilization and stimulate cell mitigation, differentiation and proliferation. During the recovery process, these scaffolds should maintain the biological and mechanical function. By the time the recovery process is ended, the scaffold should be degraded and replaced with fully functional host tissue [5], [6]. However, designing the biomaterials with desired properties which will perfectly fit their further biological function is proved to be extremely difficult and the current state of the research could be described as “a mixture of dreams and realities” [7].

The design of the functional scaffolds includes biomimetic approach. Biomaterials are produced in such a manner to imitate biologically equivalent structures as close as possible. Natural materials exhibit specific properties, which match extremely well with the biological function of the tissue they build. As an example, this was achieved by creation

of hybrid hierarchical structures spanning along multiple length scales, as in the case of bones [8] or heart valves [9]. However, mimicking the structural properties of natural materials is far from a trivial task [10], which might be related to the inability of currently employed experimental procedures for preparation of biomaterials to target hierarchical peculiarities at multiple length scales from nano to centimetre range [10], [11]. Because of that, complex implantable devices like artificial joints (hips, shoulders and knees), heart valves, stents, electronic stimulator devices (pacemakers, defibrillators, etc.) will not be replaced soon with biomimetic materials.

The number of total hip replacement (THR) and total knee replacement surgeries increased in the OECD countries for averagely 35 % and 90 % since year 2000, respectively (Fig. 1.1a) [12]. National Centre for Health Statistics (NCHS) published the comprehensive statistical data about the incidence of the THR among patients of different age in the USA (Fig. 1.1b). More than 95 % of all procedures were performed on the patients older than 45 years and the number of procedures increased from 138 700 (2000) to 310 800 (2010). The incidence among the patients from 45 to 54 years old increased for 205 % as well as their share in total number of THR surgeries (from 12% in year 2000 to 17 % in year 2010). On the other hand, the incidence among the patients older than 75 years increased for (only) 92 %, while their share among the THR surgeries decreased (from 30 % in year 2000 to 26 % in year 2010) [13].

The medical treatment of the cardiac disorders included a steep increase in the number of procedures regarding placement of cardiac implantable electronic devices (CIEDs). However, the dynamics is different among developed and non-developed countries. The number of implanted pacemakers was much higher in the developed countries. For example, 916 per million of population such treatments were performed in Germany compared to only 17 per million of population in India (Fig. 1.1c). However, the percentage increase in the number of pacemaker placements from year 2001 to year 2009 is larger in non-developed countries (Fig. 1.1d). Similar trends are observed when statistical data regarding the implantation of cardioverter defibrillators (Fig. 1.1e and f) were analysed, except that a relative increase of the performed medical procedures was also strong in the developed countries [14], [15].

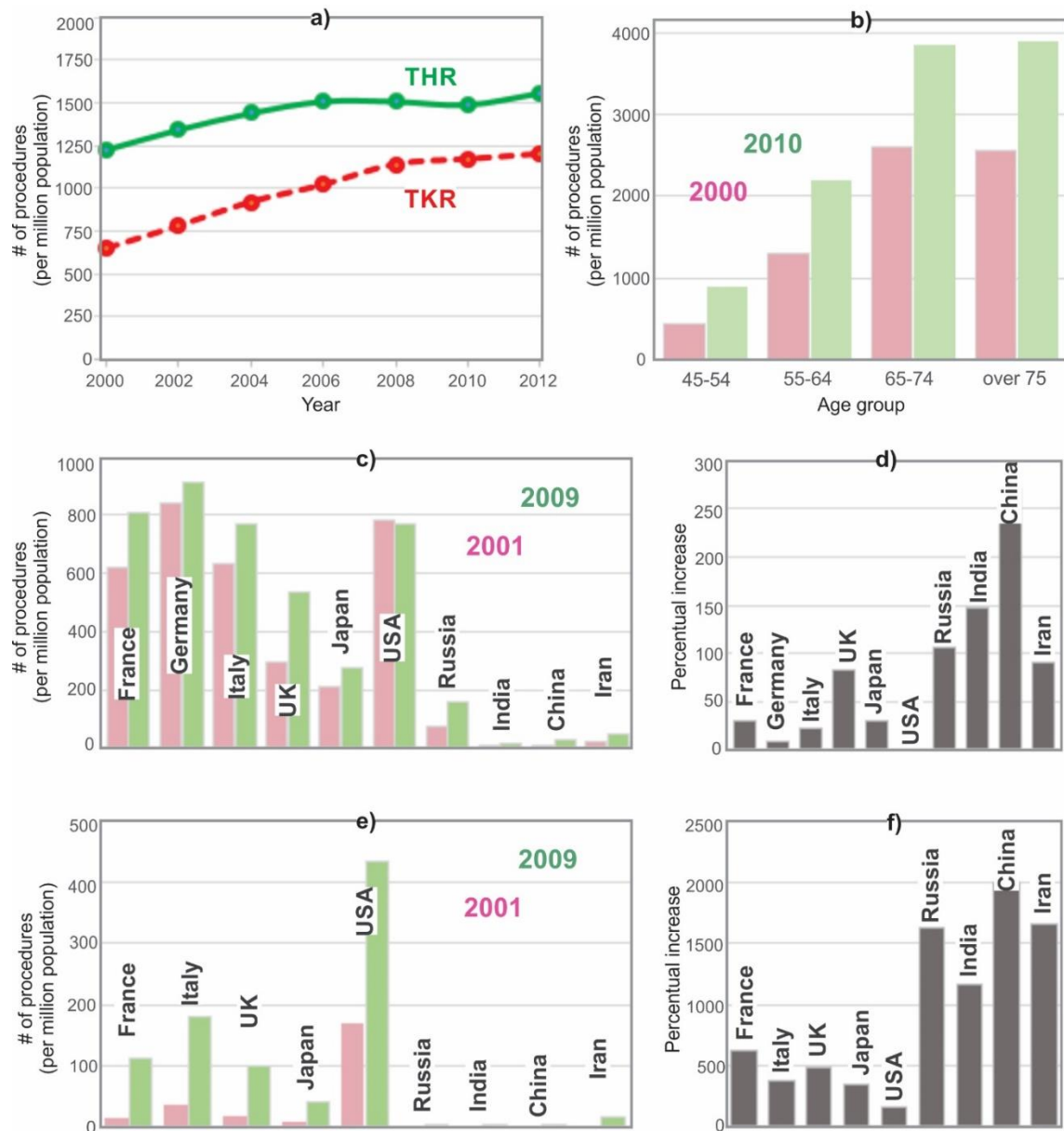


Figure 1.1: a) The total number and b) the incidence rate depend on the age of patients involved in total hip (THR) and knee (TKR) replacement surgeries [12], [13]; c) the number and d) percentage increase of implanted pacemakers form year 2001 to year 2009 [14], [15]; e) the number and f) percentage increase of implanted CIEDs from year 2001 to year 2009 [14], [15].

The data presented in Figure 1.1 strongly suggests that:

- The total number of the complex implantable device's medical procedures will increase in the future;
- The growth is expected to be faster in non-developed countries;
- The average age of patients is expected to be lower, while the average life-time is expected to be increased.

Because of the above-mentioned reasons, future prospects in functional implantable materials are oriented to designs capable to provide prolonged life-span and simpler (more economic) production.

1.2 The Host Response to the Biomaterial and Factors Causing Failure

The implanted joints and electronic devices are expected to stay within the host tissue for a prolonged period of time. The process of implant/tissue integration and reasons for possible failures will be analysed in this section. The requirements and complications of THR surgeries are used as an example to illustrate generally valid phenomena.

The implantation of a biomaterial triggers interactions with a surrounding tissue which consists of a mutually dependant cascade of events (Fig. 1.2).

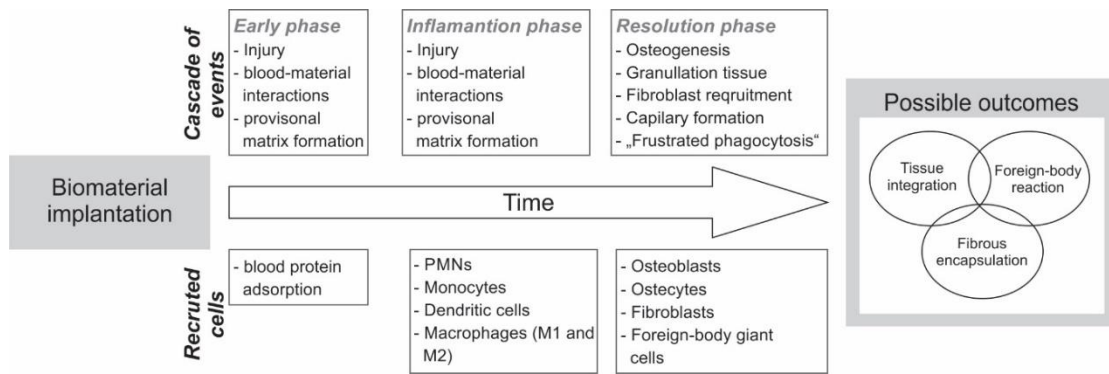


Figure 1.2: The phases of host tissue response to implanted biomaterials and possible outcomes. Adapted from [4].

In general, the placement of a biomaterial causes (micro) injury to the surrounding tissue, leading to blood flow towards the biomaterial and protein adsorption at its surface. The formed layer constitutes a temporary matrix, which acts as a transitional medium for cell/material interactions. This early stage of exposure is followed by the innate-immune response, which includes acute and chronic inflammation phase. Within minutes of implantation, neutrophils are recruited at the implantation site and their response peaks at 48-72 h after the placement of the biomaterial. They execute a signalling role via the establishment of concentration gradients of chemo-attractants, which stimulates the migration of monocytes/macrophages to the implant site [4], [16], [17]. The unwanted course of events (inflammation longer than 3 weeks) would relate to the over-excessive recruitment of macrophages, coupled with their unsuccessful phenotype change from M1 to M2, which will trigger formation of granulation tissue and fusion into foreign body giant cells. Eventually the device will fail due to the fibrous encapsulation [17]–[19]. The fast resolution of the inflammation phase (two weeks after implantation), usually means that the surrounding tissue is going to integrate well with the implanted biomaterial device. Such non-degradable biomaterials are assigned as “biotolerable” [21]. However, in practice it is hard to achieve perfect host-tissue/implanted-biomaterial contact, and foreign-body reaction is hardly completely avoided. For example, optimal osseointegration of the surrounding tissue to the artificial hip would require (i) use of unalloyed Ti implants of defined finish, (ii) implant insertion under delicate surgical procedures and (iii) *in situ* healing without mechanical loading for a period of at least 3-4 months [20].

It has been observed that high-volume surgeons and hospitals have superior outcomes, which is expressed in the reduced number of complications [21], [22]. However, they are still occurring, regardless of the taken precaution measures [23], [24]. For example, three most reported causes of THR joints complications are [24], [25]:

- Dislocations, defined as a complete loss of articulation contact between the artificial femoral head and the cup. It is mainly caused by an inadequate tissue tension or component malpositioning [25];
- Aseptic loosening, defined as a long term failure of THR due to the unresolved foreign body response. It is usually caused by wear debris, micromotion occurring in response to corrosion and oxidative reactions [26];
- Peri-implant infections, defined as a bacterial colonisation in the form of biofilm at the implant surface. They are usually caused by opportunistic bacterial species, which take advantage of the compromised body defence mechanisms at the implant-surface/surrounding-tissue interface [27].

Ultimately, dislocations and aseptic loosening could lead to an implant failure which would require a revision surgery. However, higher incidence of complications was observed after revision surgeries due to their increased complexity compared to a primary surgeries. For example, the revision of THR has 2-5 higher rates of adverse effects occurrence than the primary THR [24]. Because of that, patients might end up living with pain and impaired function rather than conducting a revision surgery when dislocations or aseptic loosening were occurring [24]. However, the revision surgeries are always performed when peri-implant bacterial infection at the surface of an implanted biomaterial is not thwarted. The infection incidences rates are published to be:

- 2,22 % in the first 10 years after THR [26];
- 1-7 % after implantation of CIEDs [25].

In the both cases, complete removal of the biomaterial is required [28], [29] as well as prolonged treatment with antibiotics is applied (e.g. 6 weeks in the case of THR [24]) before the revision surgery.

Thus, besides ensuring the satisfactory foreign body response, it is of crucial importance to completely prevent bacterial infections. In the following section challenges and current strategies for development of infection resistant surface will be presented.

1.3 Biomaterial Technologies for Infection Resistant Surfaces: Challenges and Strategies

When biomaterial is implanted within the host tissue, nearby bacteria could colonize its surface. These bacteria may either come during the surgery or by haematogenous/lymphatic seeding from a distant infected site. This process is highly favoured as bacteria have a high tendency to colonize inert surfaces of foreign bodies and develop biofilms [1]. Bacterial biofilms consist of sessile bacteria within the interconnected extracellular matrix, which enables their protection and communication. The usually involved species are *Staphylococcus aureus* and *Staphylococcus epidermidis* [30]. Bacterial biofilms are highly resistant to both, native immune system and antibiotics because of [31]:

- Failure of antimicrobial agents to penetrate the full depth of the biofilm because ICM retards their diffusion;
- Reactive oxygen species (ROS) produced by innate immune system which are deactivated in the outer layer of a bacterial biofilm, as this process is faster than their diffusion.

This implies that thwarting of a biofilm formation is essential for the prevention of bacterial infection-related biomaterials failures.

Nowadays treatment mostly consists of a systematic application of the antibiotic perioperative prophylaxis. The local antibiotic delivery is a more recent approach (e.g. delivery of antibiotics from bone cements). Both of the approaches are compromised by the problem of antibiotic resistance [32]. In general, the prevalence of resistant strains is increasing and hospitals are generally places where drug-resistant strains are found. For example, 60-70 % percent of *S. aureus* found in hospitals in the USA are multi-drug resistant, while 50 % of isolated *S. aureus* outside the hospitals already exhibit resistance to at least one antibiotic. The spreading was related to improper use in treatment of bacteria with antibiotics which also include prophylactic application of antibiotics [33]. Similarly, resistance rates of *Acinetobacter baumannii* strains isolated in one of Chinese hospitals against commonly used antibiotics were steeply increasing from 2008 to 2012 (Fig. 1.3a) [34]. In contrast, the number of newly approved antibiotics by FDA was decreased from 29 in the 80s to just 7 in the first decade of the 21st century [35]. This implies that drug-resistant bacteria would be able to come in the vicinity of the implanted biomaterial during operational procedure and cause the life threatening infection. As a consequence, revision surgery would be required.

The local delivery of antibiotics was a promising approach because it was able to provide prolonged antibiotic prophylaxis at the place of potential infection (which is in the near surrounding of the implant site). However, the residual antibiotic release was commonly observed phenomena. For example, gentamicin-release experiments confirmed the residual antibiotic release (below active concentration) from a cemented orthopaedic implant for 5 years, which resulted in the appearance of a gentamicin-resistant staphylococcal strains [32]. Thus, local delivery of antibiotics could facilitate the occurrence of resistant strains.

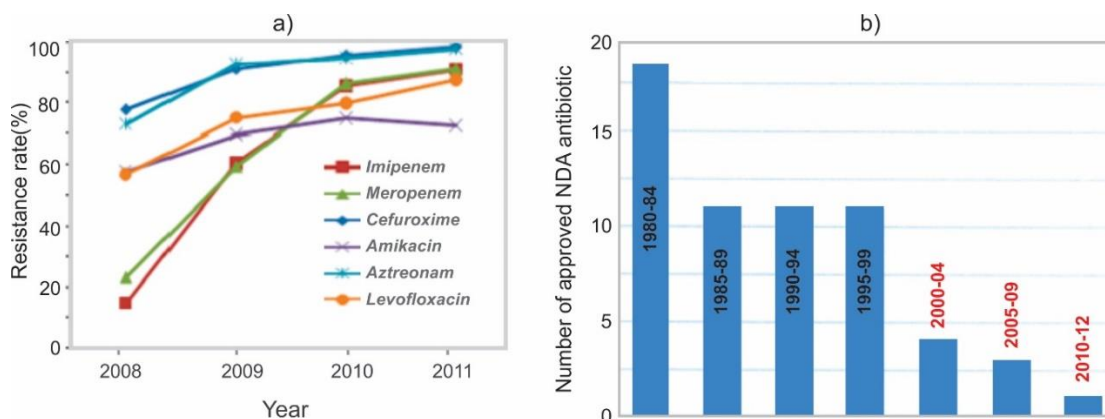


Figure 1.3: a) The increase of the resistance for *A. baumannii* against available antibiotics from 2008 to 2011 [34]; b) Time-dependent decrease of the newly approved antibiotics [35].

The previously described problems caused by the prophylactic use of antibiotics points towards an urgent need for the development of innovative strategies for thwarting biofilm formation at the surface of an implanted biomaterial. In this context, perspectives regarding

utilization of inorganic metal oxide nanostructured materials are addressed in the following section.

1.4 Antibacterial Metal-Oxide Nanostructured Materials

Nanomaterials emerged as a new paradigm to target the occurrence of drug-resistant bacterial strains [36]. The application of inorganic metal oxide (CuO, ZnO, TiO₂, MgO, etc.) particles has been favoured due to their broad spectra of antimicrobial activity. The reduction in the particle size of a material to nanometre level improves its antibacterial activity as long as the particles are well-dispersed and stable. In the case when further decrease in particle size increases their instability and consequently enhances aggregation, any further improvement in antibacterial activity fails [37]. In general, the main mechanisms of toxicity of metal-oxide nanomaterials to bacterial cells are related to [37], [38]:

- Generation of ROS species, like hydrogen peroxide and superoxide radicals;
- Release of metal ions, which could harm bacteria (e.g. Zn²⁺, Cu⁺);
- Binding to the cell wall, which could result in the damage of the bacterial wall.

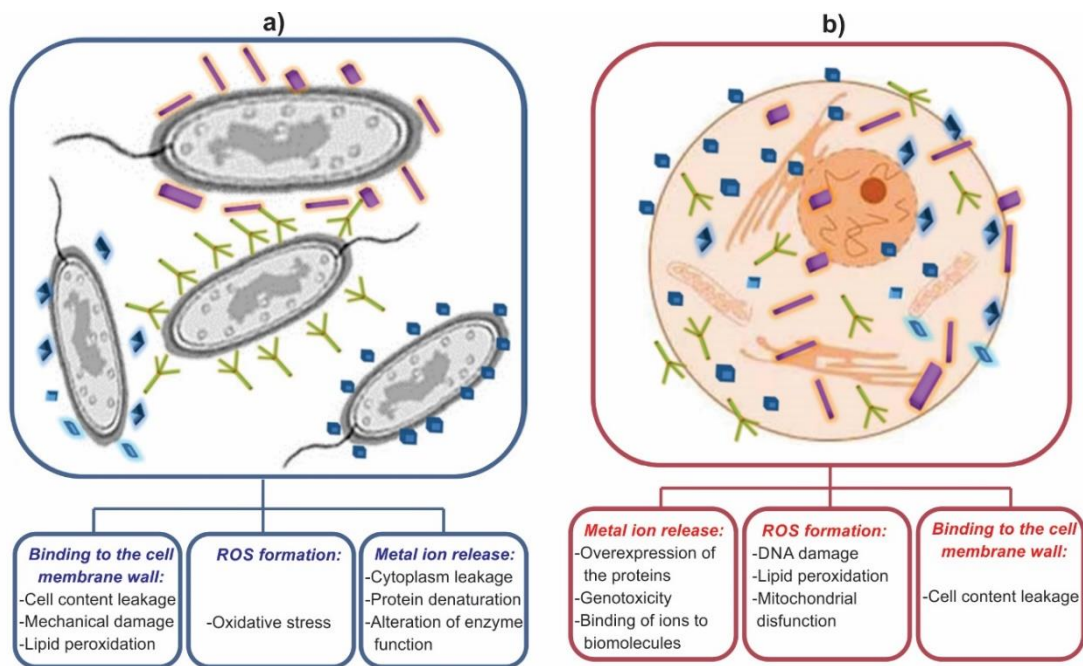


Figure 1.4: The impact of metal oxide particles on a) bacteria and b) cells [37].

Although above-listed mechanisms of antibacterial activity allow efficient elimination of the bacteria, they are non-selective and could also be toxic towards human cells. For that reason it does not necessarily mean that the materials with the most effective antibacterial activity are the best choices for preventing bacterial adhesion. It has also been shown that in some materials the trends in antibacterial activity are the same as the trends in toxicity towards the human cell. The most representative example is CuO [39] with the most effective antibacterial activity among metal oxide based nanoparticles which is also the most toxic against human cells [40]. The reason is the CuO non-selectivity due to the sameness in the mechanisms of cytotoxicity (Fig. 1.4b) and the mechanisms of antibacterial activity (Fig. 1.4a) [37], [40].

Because of the mentioned non-selectivity, the effectiveness of metal-based antibacterial nanoparticles in prevention of bacterial biofilm formation at the surface of an implanted biomaterial would have a negative influence on the host-tissue/biomaterial interactions and will contribute against successful tissue integration. This implies the need for the application of materials which would:

1. Have a potential beneficial effect on the tissue integration, while exhibiting strong and broad antimicrobial activity;
2. Exhibit different mechanisms of activity during interaction with mammalian and bacterial cells.

In the following sections, the outlooks of the vanadium pentoxide and magnesium oxide to fulfil the mentioned requirements will be evaluated. It would include an analysis of their physicochemical properties, antibacterial potential and cytotoxic effects.

1.5 Magnesium Oxide

1.5.1 Synthesis and physicochemical properties

Magnesium oxide (MgO) is a basic oxide usually synthesized by a two-step procedure. In the first step, precursor (either magnesium hydroxide [41], magnesium carbonate [42] or magnesium oxalate [43]) is precipitated from solution. In the second step, the precipitate is thermally decomposed to obtain MgO particles. The process of thermal decomposition can be performed at the temperatures higher than 350°C [44]. At lower decomposition temperatures (below 1200°C) the particle shape is retained [45]. This allows the preparation of MgO nanoparticles in the form of either agglomerated nanorods, nanoneedles, hierarchical structures or lamella-like structures [41], [42].

It is important to point out that although the shape of the particles has been retained, morphological changes of the surface were observed at decomposition temperatures (T_{calc}) above 600°C, where nano-texturing of the surface was observed due to enhanced kinetics of the MgO crystal growth [43], [46]. A comprehensive study about the influence of temperature on the nano-texturing of MgO particle surfaces was undertaken for trapezoid like particles [43]. More recently, we provided a detailed investigation in the case of MgO microrods [47]. The crystallite size of the trapezoid-like MgO microparticles produced from the $\text{MgC}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$ was increasing up to $T_{\text{calc}} = 800^\circ\text{C}$, and remained constant with the application of the higher calcination conditions (900, 1000°C) [43]. In contrast, the size of the MgO microrods' crystallites obtained from $(\text{MgCO}_3 \cdot 2\text{H}_2\text{O})$ was steadily increasing with an increase in the T_{calc} [47]. Furthermore, the trapezoid-like MgO microparticles had cracks and holes at their surface, i.e., they exhibited a pronounced porosity at the nano-scale [43]. In contrast, the prepared MgO microrods were densely packed without cracks, i.e. porosity was not observed [47]. We related the observed differences to the precursor chemical composition, making it an important parameter for the control of structural properties of produced MgO particles [47].

The MgO has been used in the broad range of application, which is closely related to its preparation conditions. For example, caustic MgO (prepared up to 100°C) [45] has been used as a catalyst while dead-burned MgO (prepared at temperatures higher than 1500°C) [45] has been utilized for production of inert crucibles for high temperature application. The reason for a different applicability is a difference in the concentration of defects at the

surface. The caustic MgO has a high abundance of defect sites and dead-burned MgO has a low abundance of defect sites at the surface [45].

The surface peculiarities of MgO were extensively studied [48]–[52]. Various possible defects were identified (Fig. 1.5) and their nature at the surface of MgO is diverse (e.g. steps, edges, kinks, vacancies, corners, etc.) [48], which results in the reduced coordination of atoms at the surface of MgO. Three coordinated atoms (3C) are observed at corners, di-step vacancies and kinks. 4C atoms are observed at the edges and around vacancies while the 5C atoms are found on terraces, compared to 6C in the bulk rock salt structure of MgO [49]. These defects, depending on their coordination and surroundings, have different electronic properties than atoms in the bulk MgO. As a result, they ionize at energies lower than the band-gap of pure MgO. During the process, exciton is created and it has been shown that it could move along the surface [50]. The correlation of electronic properties and surface coordination has been used to obtain qualitative information about the nature and abundance of various defects by means of complementary techniques: the UV/Vis reflectance, photoluminescence (PL) and electron paramagnetic spectroscopy [50]–[52].

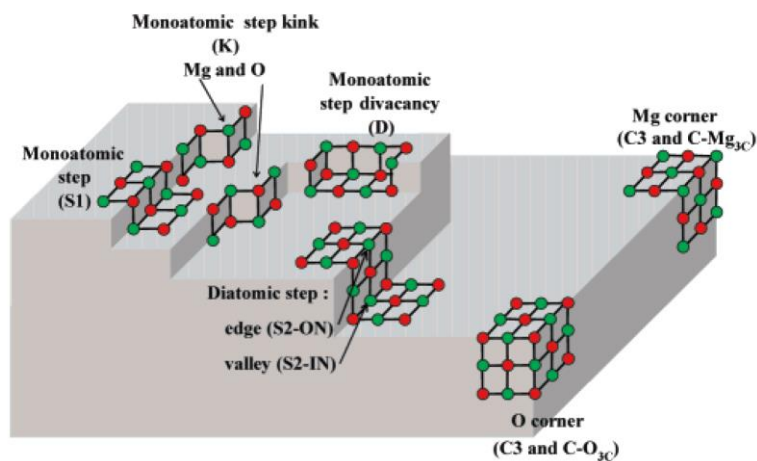


Figure 1.5: The schematic representation of various defects at the surface of MgO [49].

The reduction in the atom coordination at the surface causes surface relaxation and increase of the surface energy. It has been shown that basicity of the sites is increased by the reduction in oxygen atoms coordination [53]. This has been effectively exploited in practice to MgO improve catalytic activity, which was more efficient when basic sites at the surface were stronger [46], [54].

1.5.2 Antibacterial activity

MgO exhibited a broad range of antimicrobial activity against both, Gram positive and Gram negative bacteria. Its bactericidal potential was comparable to the bactericidal properties of ZnO [55]. It has been recognized that MgO [56]:

- Exhibits contact-based antibacterial action;
- Raises the pH in bacterial suspension due to hydration which does not contribute to its antibacterial activity;
- Dissolves in water and releases Mg^{2+} which does not cause harm to bacteria.

AFM and SEM morphology studies confirmed deterioration of bacteria wall after interaction with the MgO nanoparticles, which indicated leakage of intracellular content.

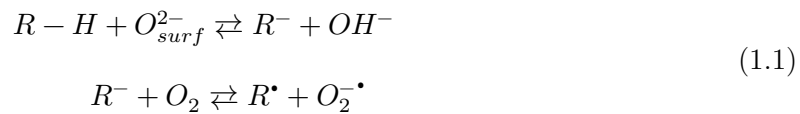
TEM study showed a lack of MgO particle internalization within bacterial, which proved that MgO particles are damaging the bacteria from the outside [57], [58].

As it defines MgO activity, the research was mainly focused on the optimization of the MgO/bacteria contact. One way to maximize the bacteria/surface contact was to use powders with a high surface-to-volume ratio, i.e., MgO in the form of nanoparticles (NPs). Huang *et al.* showed that the bactericidal potential of MgO is proportional to its specific surface area. The MgO with the highest BET exhibited the most effective antibacterial activity. This was achieved by the NPs' size reduction down to 26 nm. A further decrease in the particle size led to powders with a lower BET area, which was explained by different levels of particle agglomeration, as smaller NPs form larger agglomerates [59]. Another approach included the improvement in the effectiveness of the bacteria/surface contact by Li-doping of the MgO. The lattice replacement of Li^+ ions with Mg^{2+} ions enhanced the creation of oxygen vacancies, causing improvement of antibacterial activity. The particles were still strongly agglomerated [60].

To target the problem of agglomeration, the impact of nano-texturing on the antibacterial activity of MgO microrods was evaluated. As we have shown, preparing the nano-textured MgO microrods by thermal decomposition at 900°C provides material with reduced agglomeration, lower specific surface area and improved bactericidal potential when compared to the MgO nanoparticles [47]. The difference in antibacterial activity was contributed to a reduced concentration of non-emissive defects at the surface of nano-textured MgO microrods [47]. The observed phenomena are opposite to the trends in catalytic activity, which is improved by introduction of surface defects sites on the nanostructured MgO particles [61].

The comprehensive study on the role of the various surface defects at the antibacterial potential of MgO was not reported, except in the case of the oxygen vacancies where an electron transfer to an adsorbed oxygen molecule is possible. As-generated superoxide radical ($\text{O}_2^{\cdot-}$) was reported to be a molecular mechanism of ROS mediated MgO antibacterial activity [62]. The ROS species were either able to cause lipid peroxidation or to diffuse inside the cell and cause cell death [56], [62], [63]. However, for this to happen, energy is required to support electron transfer from vacancy towards the molecular oxygen [64]. In contrast, Leung *et al.* reported about dominant non-ROS mechanism of antibacterial activity. They explained an ability of MgO to produce ROS to the surface defects in general. They suggested polar interaction with components of bacterial cell wall (e.g. lipopolysaccharides), which cause bacterial wall disintegration and death [57]. The proposed mechanism is similar to the mechanism of activity of antimicrobial peptides [65].

Newer investigations, which are part of this dissertation, precisely determined the role of surface defects on MgO particles antibacterial activity. It has been proved that oxygen deficient MgO surfaces are more successful in the elimination of bacteria. At the same time, I demonstrated that ROS generation is rather a side product of the i) MgO hydrolysis and ii) MgO/bacteria interactions than an inherent property of MgO (manuscript submitted). This matched well with the ability of MgO to generate O_2^- ions via the action of presorbed organic molecules (R-H) [64] according to Eq. 1.1.



1.5.3 Toxicology

Magnesium is the second most abundant intracellular cation and the fourth most abundant cation in the human body [66]. The normal human body contains around 22-26 g of magnesium. It is located mainly in the bone, muscle, soft tissue and extracellular fluid (Fig. 1.6). Approximately 20-30 % of magnesium found in tissues is readily exchangeable. Less than 1 % of magnesium is located in extracellular fluid, where it is either bound to proteins, ionized or complexed with various anions (e.g. phosphate, citrate, etc.) [66].

The recommended daily allowance for magnesium in adults is 4.5 mg per kg of body weight. It is closely related to Mg concentration in drinking water and nutrition. It is plentiful in green leafy vegetables, cereal, grain, nuts and legumes. However, refining and cooking of the food reduce the absorbable magnesium content by nearly as 85 %. The consumed Mg is absorbed in intestine (Fig. 1.6) and the fractional resorption depends on the intake: up to 65 % at low intake and as low as 11 % at high intake. Although the roles of parathyroid hormone and vitamin D were suggested, the controlling mechanism of Mg absorption was not completely revealed [66]. The Mg homeostasis depends on the kidney function, where most of the filtered Mg in the blood is reabsorbed (Fig. 1.6) [66].

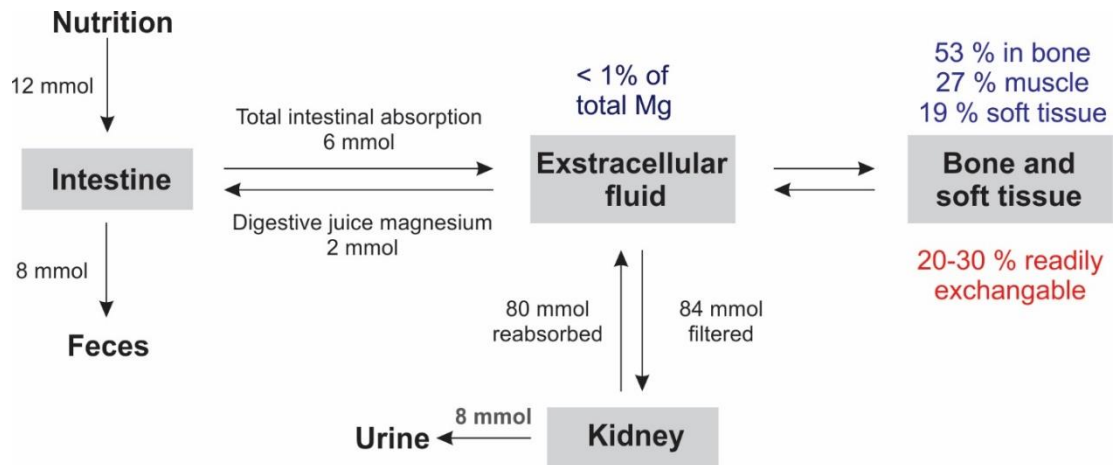


Figure 1.6: The metabolism and storage of Mg in human body [66].

In the human body Mg^{2+} cation exhibits the abilities to [67]:

- Form chelates with anionic ligands;
- Compete with Ca^{2+} for binding sites on proteins and membranes, ensuring the low free calcium intracellular levels.

As a result, its role is essential in many physiological processes like enzyme activity, membrane processes, functioning of muscle and neural tissue and formation of bone and dental tissue, which implies necessity of the efficient Mg balance (Fig. 1.7) [66].

This implies necessity of balance of magnesium levels in the human body. The Mg^{2+} deficiency is seriously affecting cell homeostasis, which could manifest in various clinical conditions. In this work, we were focused on an impact of reduced Mg^{2+} levels on the processes, which are relevant to host-tissue/implanted-biomaterial interactions, i.e. bone tissue (joint replacement), cardiac tissue (CIEDs) and inflammations. For more general information, a literature review is available [66].

Mg^{2+} deficiency and osteoporosis were related in the past [68]. The low serum levels require Mg release from the storage, i.e. bone resorption. However, Mg does not have a passive role inside the bone as it is critical for new bone formation. It is included in

synthesis of hydroxyapatite particles of proper size and crystallinity. In the case of Mg deficiency, newly formed crystallites are larger and better structured. Experimental studies in rodents related decreased bone formation to reduced osteoblastic activity and pronounced osteoclastic activity. In contrast, high levels of Mg^{2+} hinder the hydroxyapatite formation and result in the formation of enzymatically non-degradable phosphate phase. Thus, regulation of Mg^{2+} concentration is critical for both, new bone formation and healthy bone tissue [68].

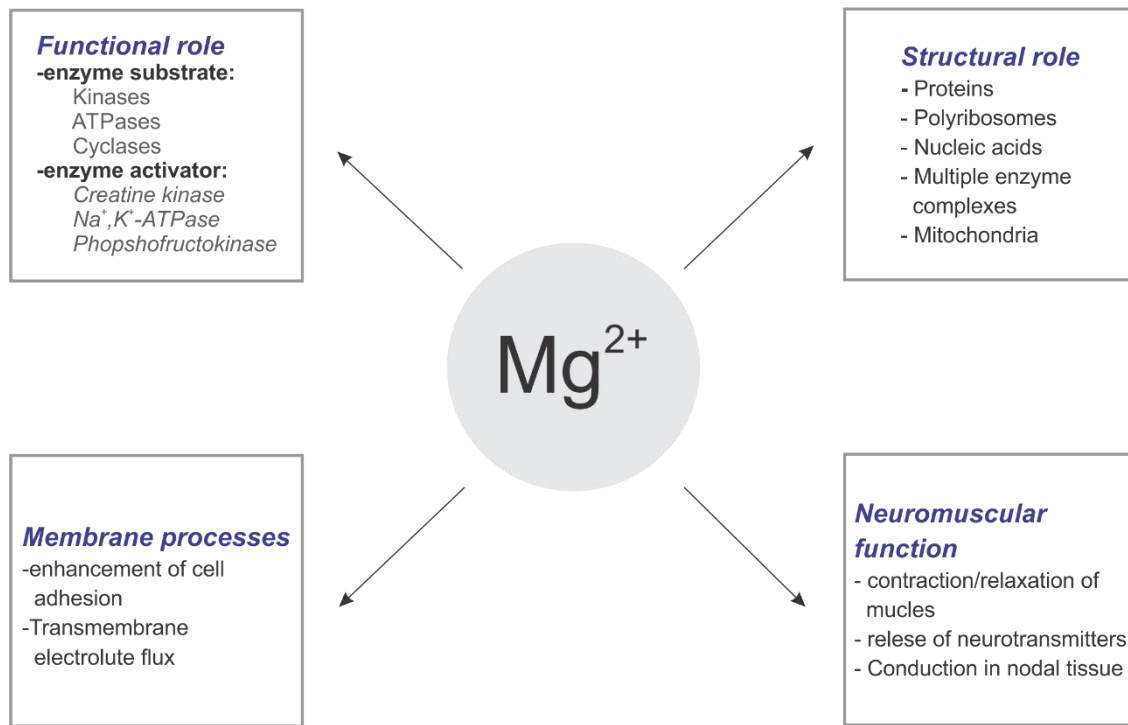


Figure 1.7: The impact of magnesium cation on various cellular processes and structures. Adapted from [67].

Mg^{2+} deficiency (serum concentration below 0.5 mmol/L) could affect cardiac electrical activity, myocardial contractility and vascular tone. It has been linked to atherosclerotic cardiovascular diseases, which was achieved by magnesium impact on lipid metabolism, platelet aggregation and blood pressure increase. In contrast, elevated levels of Mg^{2+} (2-3 mmol/L) could cause hypotension and reduction in skeletal muscle function while extremely high levels (7 mmol/L) are lethal due to complete heart block. Thus, optimal function of heart and blood vessels is conditioned by successful regulation of Mg^{2+} levels [66].

Inflammation is also affected by Mg deficiency. *In vivo* tests on rats demonstrated that Mg deficiency caused [69]:

- Elevation of plasma pro-inflammatory cytokines (e.g. interleukin-6 and circulating substance P). The production of tumor necrosis factor alpha was enhanced only after an immune stress;
- Enhanced tissue, erythrocyte and lipoprotein peroxidation, oxidative modification of proteins, reduced antioxidant status and increased plasma nitric oxide. These suggested an increase in ROS generation;

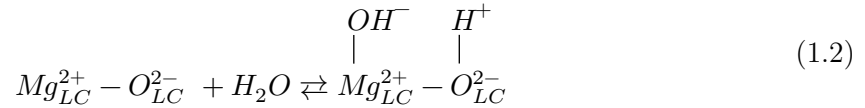
- Increased recruitment and activity of phagocytic cells.

One of the possible sources of magnesium could be MgO. The clinical study about its applicability was performed and has shown positive effect on the reduction of hypertension, without any harmful effects reported. The daily intake was 1g for 21 days [70]. In contrast, *in vitro* studies pointed out toxic effect of MgO on human cells [71], [72]. The authors suggested a narrow therapeutic window where MgO would have a positive effect on osteoblast activity while still providing antibacterial protection [72]. Thus, the future development of MgO delivery systems is a matter of necessity.

Based on the overview of MgO antibacterial mechanisms of activity and biological role in human body, we concluded that a solid state is required to eliminate bacteria while ionic form could act beneficially in the stimulation of cell activity, tissue integration and reduction of pro-inflammatory response. However, it is important to consider hydration kinetics of MgO solid which provides release of Mg^{2+} ions, as elevated Mg^{2+} levels could negatively impact the process of host-tissue/biomaterial-surface integration.

1.5.4 Hydration and dissolution

MgO exhibits a high tendency to interact with water. When exposed to air, it tends to adsorb water molecules at the surface following Eq. 1.2 [73]:



The coordination of surface atoms (Mg and O) was determining:

- The bonding energy of water molecules. It was stronger when water was adsorbed at the low coordinated sites (kinks, dislocations, etc.) [49];
- Bonded hydroxyl groups were either isolated or were forming mutual hydrogen bonding. Because of that, IR spectrum of MgO could exhibit several different absorption bands [74].

MgO particles react with water, i.e. hydration occurs causing the growth of magnesium hydroxide ($Mg(OH)_2$). The observed reaction is exothermic [75]. Thermodynamically most stable crystal planes of MgO in gaseous atmosphere are $\{100\}$ planes, which suggests growth of MgO cubes in ideal conditions. In contrast $\{111\}$ planes of MgO are the most stable in aqueous environment. This indicates necessity of MgO surface reconstruction and explains hydrolysis of MgO [76]. However, MgO/water interface is strongly controlled by kinetic factors. It has been shown that hydration of ideally epitaxial grown (100) MgO crystals did not proceed at all, which indicated MgO dissolution rather along than perpendicular to (100) planes. In contrast, highly defected surfaces and particles were readily dissolved, which confirmed the importance of low-coordinated MgO surface sites during hydration [77]. In line with this study was the observed degradation of MgO cubes preferentially via cube corners. It was argued that each removal of water molecule increases the number of low coordinated sites. For example, at the beginning in the corner of MgO cube only one 3C oxygen atom is situated. When two and nine MgO molecules were dissolved, two and four 3C oxygen atoms are observed, respectively (Fig. 1.8) [78]. As a result, the MgO dissolution curve had a sigmoidal shape over time, i.e. reaction kinetics in the initial stage was progressive [79].

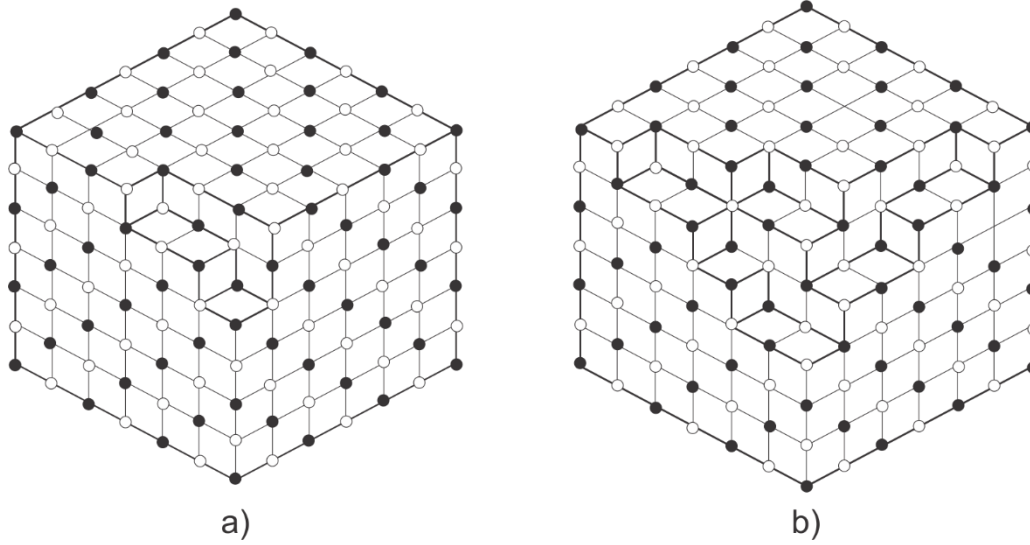
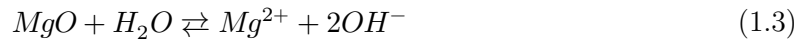


Figure 1.8: An idealized MgO cube after removal of a) two and b) nine water molecules, respectively [78].

The dissolution process for MgO depends on the pH of the aqueous solution. When $\text{pH} > 7$, the MgO dissolution follows the chemical reaction, according to Equation 1.3 [79].



The rate determining step includes hydroxyl absorption at the surface of MgO. After the supersaturation, precipitation of $\text{Mg}(\text{OH})_2$ was observed at the surface of MgO [79]. This indicated the epitaxial growth of $\text{Mg}(\text{OH})_2$ on MgO [79], which could be explained by topotactic relationship between (111) planes of MgO and (0001) planes of $\text{Mg}(\text{OH})_2$ [77].

The formation of $\text{Mg}(\text{OH})_2$ from dissolved MgO could be decelerated by the action of various anions by either:

- Complexation of released Mg^{2+} ions (e.g. using EDTA) [80];
- Formation of protective layer at the surface of MgO (e.g. chloride, sulphate, citrate, etc.) [80], [81].

In contrast, the addition of a common ion (either Mg^{2+} or OH^-) enhanced the nucleation and crystal growth of $\text{Mg}(\text{OH})_2$ [81].

The interaction of MgO with cell growth media (either for mammalian or bacterial cells) provided interesting behaviour. When mixed with Luria-Brentani (LB) broth media, MgO was completely transformed to $\text{Mg}(\text{OH})_2$. In contrast, interaction with blood proteins from Fetal bovine serum (FBS) inhibited the transformation and no traces of $\text{Mg}(\text{OH})_2$ were found in the mixture after 24 h. This was explained by a formation of protein corona at the surface of MgO, which prevented topotactic growth of magnesium hydroxide. However, the proteins from LB media were not able to form an effective barrier and thwart the dissolution of MgO and subsequent precipitation of magnesium hydroxide [82].

In the context of coupling MgO antibacterial and bioactive properties, its dissolution and possible magnesium hydroxide formation are important factors to consider. The MgO application requires design of a delivery system capable of controlling MgO hydration and dissolution which is investigated in detail within this dissertation.

1.6 Vanadium Pentoxide

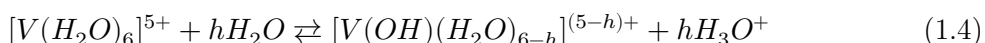
1.6.1 Synthesis and physicochemical properties

Vanadium pentoxide has a layered crystal structure (Fig. 1.10a), and the unit cell is orthorhombic (principal axis: $a = 11.555 \text{ \AA}$, $b = 4.371 \text{ \AA}$, $c = 3.557 \text{ \AA}$). The chemical bonds are observed in x and y dimension, while in z direction Van der Waals forces are keeping the structure together. Vanadium has partially filled d -orbitals, which are responsible for a wide variety of electronic, magnetic and catalytic properties. The layered structure can explain a rather low melting point of vanadium pentoxide (670°C) [83]. Considerable extension of vanadium's d -wave functions (which are not involved in the metal-oxygen bonding) with respect to the equilibrium distance between neighbouring atoms allows an overlap with the p -orbital of π -symmetry. As a consequence, the degree of charge separation between V and O is reduced. The presence of large displacement polarizabilities reduces the cation – cation repulsions, enabling shorter metal-metal distances [84].

Vanadium pentoxide based 1D nanostructures were produced on the large scale using a hydrothermal method by the application of different precursors which resulted in:

- vanadium pentoxide nanowires (formed from either vanadium peroxovanadate [85] or vanadyl-sulphate/potassium-bromate mixture [86]);
- water intercalated either nanoribbons (formed from peroxovanadate) [85], [87] or nanobelts and nanorolls (formed from acidified ammonium metavanadate solution) [88];
- alkali-metal intercalated nanoribbons (formed from vanadium pentoxide/alkali solution) [89].

The underlying mechanism of growth was related to a strong polarizing power of the small and highly charged V^{5+} cation. When present in water, it is strongly hydrolysed by surrounding water molecules following the reaction as presented in Equation 1.4.



The equilibrium strongly depends on the pH and temperature. Hydrolysis ratio (h) varies from 4.5 up to 6.2. At room temperature, the coordination number of water/hydroxyl molecules decreases from 6 to 4 when pH is above 6. The reason is transfer of σ electron from the coordinated water molecules toward the empty d -orbitals of V^{5+} ions, which is more pronounced with deprotonation, leading to a decrease of the positive partial charge of vanadium [90]. As a consequence, V–O bonds become more covalent and vanadium coordination decreases to four-fold coordinated vanadate species. This phenomenon causes an increase in the splitting of d -orbitals and can be seen with the naked eyes as 6-fold coordinated decavanadate ions are orange while 4-fold coordinated metavanadate ions are colourless [90]. Monomeric species are observed only in very dilute solutions (Fig. 1.9a).

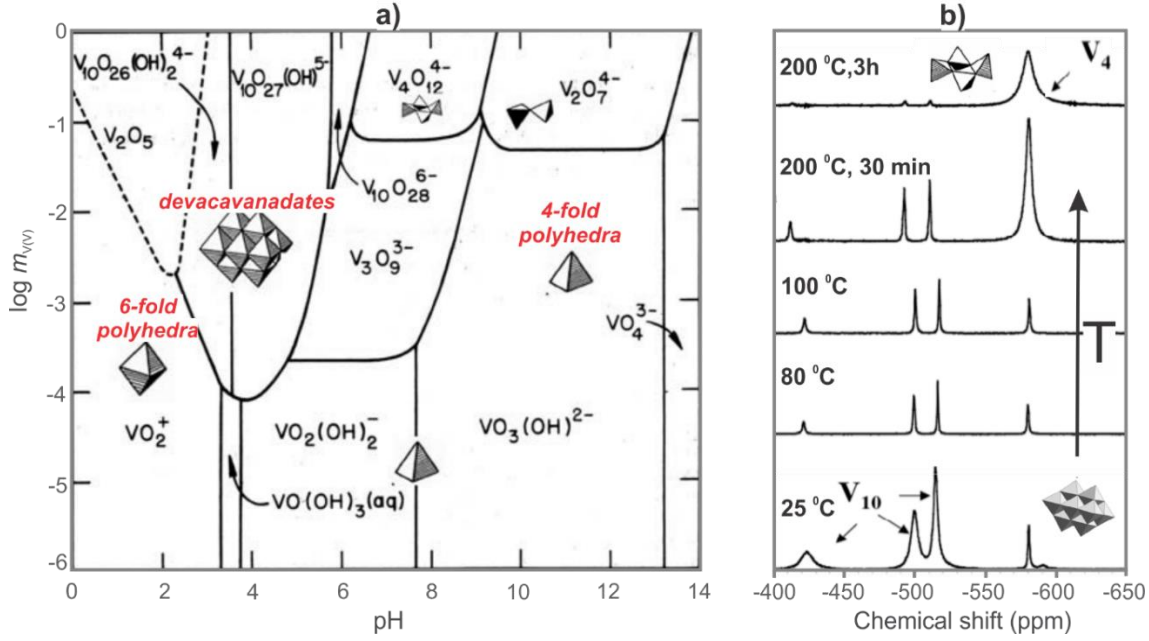
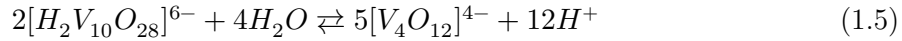
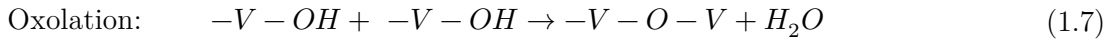
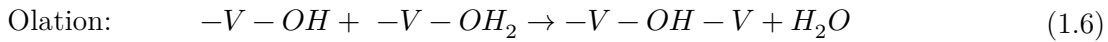


Figure 1.9: The molecular structure of V^{5+} /water complexes in aqueous media depending on a) pH, concentration and b) temperature. Adapted from [90].

Similar changes from 6-fold to 4-fold coordinated vanadium were observed during hydrothermal synthesis. Above 200 °C, only metavanadate forms are observed (Fig. 1.9b) as deprotonation occurs upon heating, resulting in the dissociation of decavanadates into metavanadates, according to Equation 1.5 [90].



The rising of the vanadium concentration in aqueous solution will trigger the start of the condensation reaction. Two main reactions involved in this condensation process are olation and oxolation, according to Equations 1.6 and 1.7, respectively [90].



Both mechanisms involve the nucleophilic addition of negatively charged OH^- groups onto positive vanadium cations. The reaction occurs only x - and y -directions while it is not observed in z -direction. Olation reactions progress via labile bonded water molecules, making their rate faster than the rate of oxolation reactions. As a consequence, various modes of anisotropic growth might be observed [90].

In aqueous solution (free from metal ions), condensation causes the formation of bi-layered sheets of V_2O_5 polyhedra (Fig. 1.10a). These layers stack to form nanoribbons, with water molecules in between. The amount of intercalated water molecules was varying, depending on the process conditions [91]. The layers were not stacked in perfect registry, but were rather turbostratically disordered [91] leading to the formation of vanadium pentoxide xerogel ($V_2O_5 \cdot xH_2O$). For example, this compound was obtained by hydrothermal treatment of peroxovanadate precursor up to 160 °C [85].

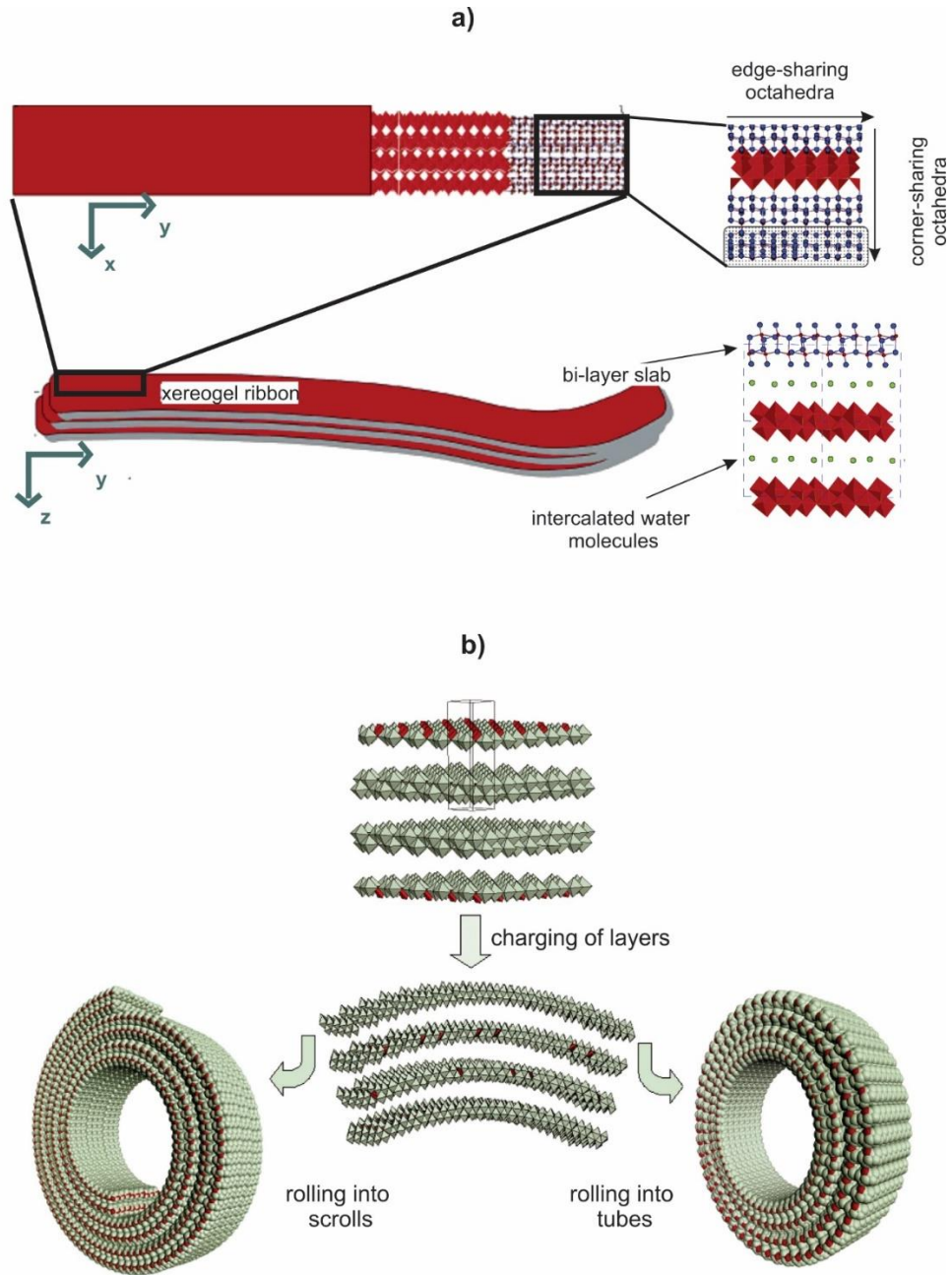


Figure 1.10: The schematic presentation of the mechanism of a) vanadium pentoxide xerogel formation [91] and b) vanadium pentoxide nanotubes rolling [92].

The increase in the processing temperature (e.g. 180°C) would lead to the formation of orthorhombic V_2O_5 nanowires. At these conditions, bi-layered sheets rather roll than stack (Fig. 1.10b). As already observed in the case of TiO_2 [93], the reason could be charging which prevents their stacking [92],[93]. The simulation suggests that formed structures have at least 10 nm in diameter [92].

The presence of metal ions in reaction could cause their intercalation within the bi-layer V_2O_5 slabs and formation of vanadium pentoxide bronzes. The conductivity of pure vanadium pentoxide is low and intercalation increases conductivity. The structure is monoclinic and chemical bonding can be observed in third dimension, in contrast with pure vanadium pentoxide [94].

Table 1.1: The amount of intercalated metal ions and distance between V_2O_5 layers for various $M_xV_2O_5$ [94].

Intercalated atom (M)	Na^+	Ag^+	Ba^{2+}	Ni^{2+}	Al^{3+}	Fe^{3+}
x	0.33	0.33	0.16	0.16	0.11	0.11
d (Å)	10.9	11.3	11.2	13.4	14.2	14.1

The charge compensation is achieved by partial oxidation of V^{5+} to V^{4+} . Other monovalent ions, like Na^+ , K^+ etc. form compounds with a similar structure. Intercalation was also observed for di- and trivalent ions. In Table 1.1, inter-lamellar distances between V_2O_5 layers and amount of inserted ions are summarized [94]. The distance between V_2O_5 layers was increasing with the size of an anion (e.g. Na^+ vs. Ag^+) and with its charge (e.g. Na^+ vs. Al^{3+}) [94].

1.6.2 Catalytic properties and antibacterial potential

Vanadium pentoxide is a potent oxidation catalyst, which is extensively utilized in industrial processes for [95]:

- Transformation of sulphur dioxide to sulphur trioxide;
- Oxidation of naphthalene to phthalic anhydride;
- θ -xylene to phthalic anhydride transformation;
- Selective oxidation of alkyl pyridines to nicotinic acid;
- Oxidative destruction of chlorinated hydrocarbons, etc.

The ability of V_2O_5 to catalyse various oxidation reactions was related to its possibility to compensate a removal of oxygen atoms from the structure by facile rearrangement of coordination polyhedral from a corner-linked into an edge-linked arrangement [84]. Among other reactions, recent studies have highlighted the ability of nanostructured V_2O_5 to mimic the myeloperoxidase activity [96], [97]. Myeloperoxidase is an enzyme that is present in human neutrophils. Its role is to eliminate bacteria via the catalysis of the hydrogen-peroxide-to-hypochlorite transformation in the presence of chloride ions, according to Eq. 1.8 [98].



There have been no reports about the utilization of V_2O_5 for antimicrobial protection in medicine, but the previously mentioned biomimetic property of V_2O_5 was effectively utilized for the processing of an anti-biofouling ship-hull coating [97]. It was argued that the sea water represented the source of hydrogen peroxide, as its concentration in sea water is around 100 nM [97]. However, it has been shown that vanadium pentoxide generates reactive-oxygen species (ROS) on its own [99]. The latest studies (which will be presented in this dissertation) proved that V_2O_5 is able to harm bacteria only in the presence of chloride ions. At the same time, the activity was observed either with or without added hydrogen peroxide. Because of that, we proposed a two-step mechanism of vanadium pentoxide antibacterial activity, which includes [100]:

- Generation of ROS in the first step and

- Transformation of the generated ROS to antibacterially more potent hypochlorite ions in the second step.

These are the first investigations on designing properties of nanostructured V_2O_5 and their antibacterial activity for the application in biomedicine.

1.6.3 Toxicology

Vanadium is present in many different mineral forms in the earth's crust. Natural release to the environment is occurring in the processes of weathering of soil and rock, continental dusts, marine aerosols and volcanic activity. However, release from industrial sources involved in fuel combustion contributes to environmental vanadium levels to a greater extent than natural sources. Thus, inhalation exposure to vanadium is most important [101]. Cytotoxicity induced by vanadium compounds was well documented and for that purpose, Occupational Safety and Health Administration (OSHA) has set 0.05 mg/m^3 to be an exposure limit for vanadium pentoxide dust present in workplace air for an 8h workday, 40 hours per week [99].

In her PhD thesis, K. Fisher extensively studied the impact of vanadium pentoxide on the alveolar epithelial cell line A459 and determined [102]:

- Vanadium uptake by cells was linearly proportional to solubility of vanadium pentoxide;
- Low vanadium pentoxide concentrations stimulate proliferation;
- Acute toxicity (MTT assay) of vanadium pentoxide was dose-related. At low concentrations ($2 \text{ } \mu\text{g/cm}^2$), metabolic activity of cells was increased (due to proliferative effects). At higher concentrations ($20 \text{ } \mu\text{g/cm}^2$), their metabolic activity was reduced;
- The negative impact of vanadium pentoxide to membrane integrity of cells (LDH assay) was observed at high vanadium pentoxide concentrations ($20 \text{ } \mu\text{g/cm}^2$);
- Generation of ROS was observed just at vanadium pentoxide concentration above $10 \text{ } \mu\text{g/cm}^2$;
- The absence of genotoxicity of nanostructured vanadium pentoxide, while weak genotoxic potential of vanadate ions.

Thus, vanadium pentoxide toxic effects are dose dependent and might be related to its dissolution to vanadate ions [102]. These ions could exhibit a bi-phasic nature, i.e. at low concentration could enhance cell proliferation and metabolic activity while at higher concentration levels these ions exhibit cytotoxicity [102]–[104].

The molecular mechanisms of vanadate impact on cellular processes were mainly related to vanadate-phosphate analogy as phosphates have a central role in biology. As a result, vanadates are able to inhibit various enzymes [105]:

- Phosphatases (e.g. K^+ , Na^+ -ATPase, acid phosphatases, protein phosphatases);
- Ribonucleases;
- Phosphorylases.

Therefore, they are assigned as “imperfect transitions state analogue” of phosphate ions. The inhibition of these enzymes could lead to cell-cycle arrest. The protective mechanism includes a reduction of V^{5+} (vanadate) to V^{4+} (vanadyl) by intracellular antioxidants [105].

It has been shown that both, vanadate and vanadyl ions, were able to elicit similar proliferative response of fibroblasts (Swiss3t3 cell line) in the concentration range from 2.5 μM and 10 μM (Fig. 1.11). This could be related to the ability of vanadium compounds to exhibit “insulin-mimicking”, which increased the cellular uptake of glucose [106].

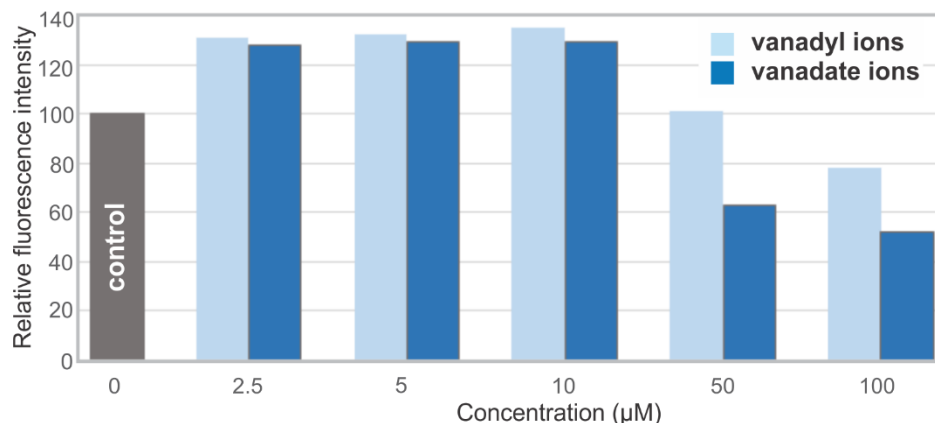


Figure 1.11: The cell proliferation as a function of either vanadyl or vanadate concentration. Adapted from [103].

However, exposure of cells to higher ions concentrations resulted in the differences among the tested ions. Vanadyl ions at concentrations of 50 μM and 100 μM reduced the cell proliferation for 1 and 22 % when compared to the control, respectively (Fig. 1.11). Vanadate ions exhibited stronger cytotoxicity, as cell proliferation was reduced for 38 and 47 %, respectively (Fig. 1.11) [103]. This implies that at concentrations higher than 10 μM , vanadates influx into the cells is too burdensome for cellular metabolism to reduce vanadates in vanadyl ions.

The vanadates (VO_4^{3-}) enter the membrane through the anionic channels (Fig. 1.12). Their reduction to V^{4+} allows prevention of phosphate-enzyme inhibition, but vanadyl cations are involved in Fenton-type reaction in the presence of hydrogen peroxide which results in the creation of hydroxyl radical and vanadate ions. Another reaction pathway includes reaction with superoxide ions, where peroxovanadyl complex is created. Its reaction with NADH would end in the formation of vanadates and hydrogen peroxide as a side product (Fig. 1.13). However, the superoxide-related reaction sequence is occurring only at elevated concentrations of vanadate ions (higher than 25 μM) [107].

In-vivo studies of rats proved that orally administrated vanadates stimulated the orientation of the fibroblasts in parallel arrays early in the tissue-repair process, i.e., vanadate ions can accelerate tissue repair [108]–[110]. Further studies showed that vanadates improved the bone-formation rate, the mechanical strength and the mineralization [111], while the pro-oxidant potential of vanadates was not revealed in erythrocytes [112]. These studies confirmed the potential bioactive role of vanadate ions when they are properly delivered. Our opinion is that equilibrium solubility of vanadium pentoxide (1-8 g/L) which is three orders of magnitude higher than vanadates bioactive range (2-10 μM), means that the design of effective delivery control of vanadium pentoxide is crucial for its application in medicine.

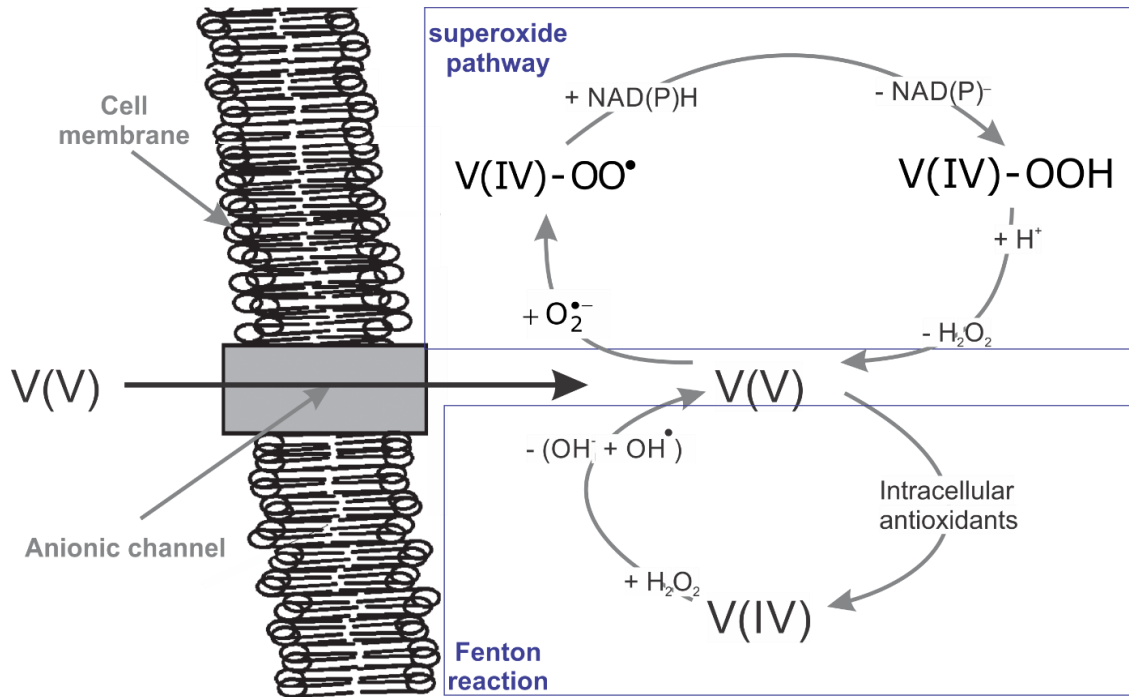


Figure 1.12: The uptake of vanadate ions in cells via the anionic channel and their biochemical reactions within the cells [107].

Based on the reviewed literature we came to the conclusion that both, MgO and V₂O₅, require to be in the solid state to eliminate bacteria while ionic form (if efficiently delivered) could act beneficially in stimulation of cell proliferation activity and acceleration of both, soft and hard tissue repair. In the following section, the principles of controlled delivery from nanocomposite coatings systems will be discussed.

1.7 Release of Either MgO or V₂O₅ from Composite Coatings

Nanotechnology provides powerful tools for designing and processing of multifunctional composites for application in medicine. One approach includes the preparation of inorganic composites. For example, inorganic TiO₂ matrix was used to stabilize V₂O₅ in the form of a sol-gel processed composite bio-coating at the implant surface. As-prepared coating exhibited controlled release of vanadate ions within a bioactive concentration range. Because of that, cell adhesion to the coating surface was stimulated. However, the delivery of vanadates was not sustained. The amount of dissolved vanadium pentoxide from the coating was 1.5 % after 3 days and below 3 % after 30 days [104]. As a result, long-term cytotoxic issues could be possible.

Another approach includes embedding of nanoparticles inside a polymeric gel or matrix. The gel/matrix serves as a carrier of particles, while ceramic nanoparticles were providing function. It could be either antimicrobial, bio-active, conductive, etc. [113]. The applied polymers were either from a natural source (e.g. collagens, chitosan, silk, etc.) or synthesized in a laboratory (e.g. polyesters, copolymers) [114].

Natural source polymers are biodegradable and biocompatible. Collagen is a constitutional part of both, soft and hard tissue. Its biocompatibility and bioactivity, like stimulation of cell proliferation and differentiation have been proven [114]. Apart from biodegradability and biocompatibility, chitosan also exhibits antimicrobial potential [115].

Aliphatic polyesters such as polyglycolic acid (PGA), polylactic acid (PLA) and polycaprolactone (PCL) are the most commonly used artificial polymers for tissue engineering applications. Their degradation products are present in the human body and can be removed by natural metabolic pathways. Their processing was done by the means of various methods (e.g. solvent casting, spin-coating, sonochemical precipitation, electrospinning of fibres, etc.). Especially attractive are their co-polymers due to highly controllable physicochemical properties. Examples are their mechanical and degradation properties which could be influenced by regulation of molecular weights, block structures, monomer rations, degradable linkages, etc. [114].

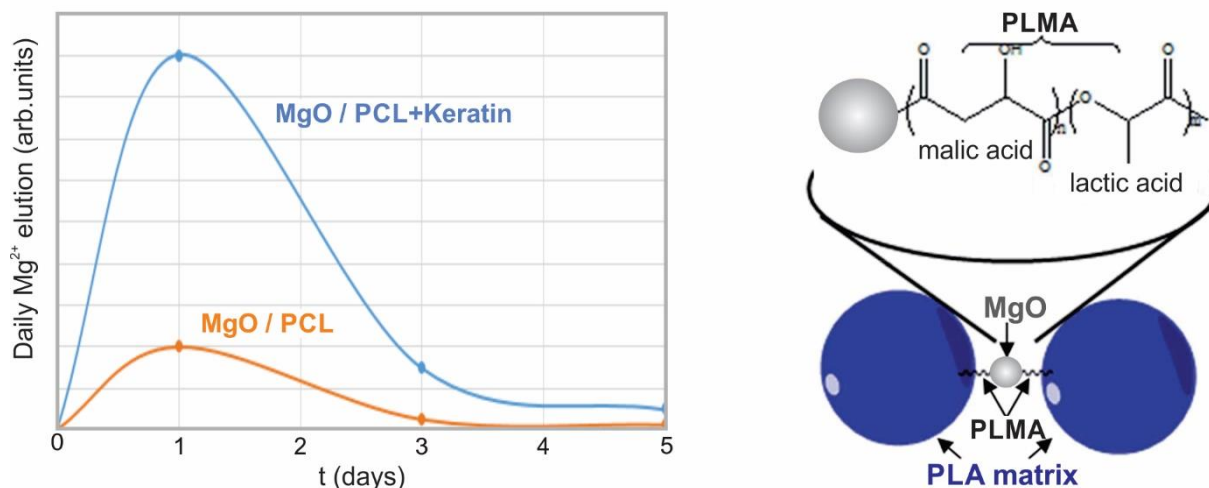


Figure 1.13: a) The daily elution of magnesium ions from different MgO/PCL composites [116]; b) Schematic representation of malic acid role in the stabilization of MgO/PLA composites [117].

Literature does not include many examples of the MgO/polymer composites made as systems for control release of Mg^{2+} ions. One of the examples is the MgO/PCL composite for the controlled delivery of bioactive Mg^{2+} [116]. It has been shown that 1:1 ratio (50 % of MgO) among inorganic and organic component can seriously deteriorate the tensile strength at break, compared to pure PCL. The release of ions showed a strong initial-burst effect, i.e. pronounced release of the component in the early stage of exposure (Fig 13a) [116]. In contrast, the addition of a small portion of MgO (1%) to PLA resulted in improvement of the mechanical properties [117].

The observed phenomena were related to the weak interaction of MgO particles (polar component) and either PLA or PCL (non-polar component) within MgO/polymer composites. An introduction of polar polymer (e.g. keratin) was reported to improve the mechanical characteristics of MgO/keratin/PCL composites when compared to MgO/PCL composites [116]. The fast degradation of the keratin stimulated the release of Mg ions. However, the problem of the initial burst effect was not solved (Fig. 1.13a) [116]. An introduction of a malic-acid/lactic-acid mixture (PLMA) as a surface active agent was reported to improve the interfacial interaction between polar MgO and PLA (Fig. 1.13b) [117]. The malic acid was adsorbed at the surface of MgO and via ester linkage to lactic acid grafted to the surface of PLA. As a result, mechanical properties were improved compared to MgO/PLA composites, which was related to a more uniform distribution of particles within the PLA matrix [117]. Thus, the modification of inorganic-particle/polymer-matrix interactions and the particle concentration strongly affected the physicochemical properties of the composites.

The application of either V₂O₅ or MgO within polymer-based coatings would require a strict control of their solubility and delivery of vanadate and magnesium ions, respectively. In general, the solute release kinetics is governed by a mutual interplay of factors related to properties of a polymer matrix, a release medium and solute properties (Fig. 1.14) [118]. In reality, the following examples could be possible [118]:

- Example 1: If polymer composition includes degradable bonds, enzymes present in the medium or cellular systems could enhance its degradation which increases the amount of released solute over time;
- Example 2: If solute compound forms strong interactions with a polymer matrix, its diffusion towards the medium would be hindered which implies reduced release kinetics;
- Example 3: If the polymer matrix is swelling (absorbs a lot of water), than the effect of strong matrix/solute interactions is erased; etc.

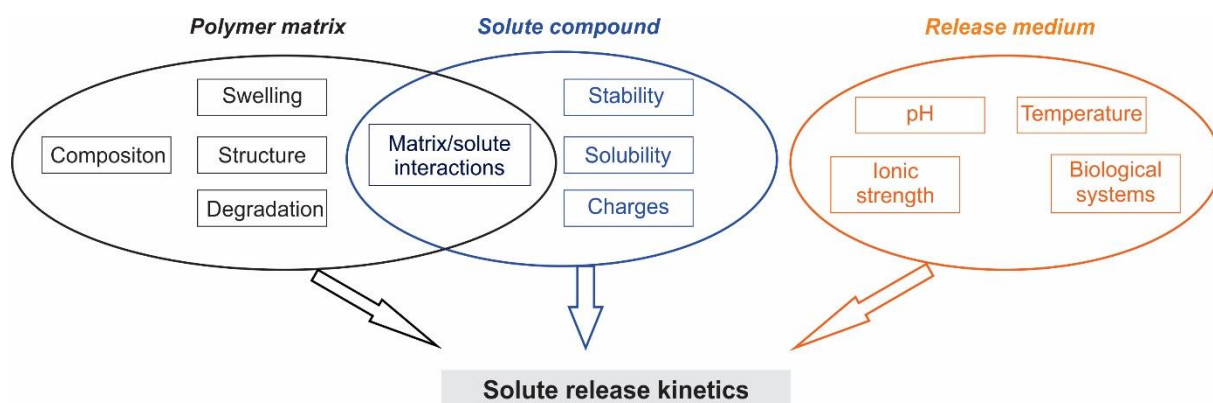


Figure 1.14: Schematic representation of factors influencing solute release kinetics. Adapted from [118].

All of the influencing factors would not contribute equally to the solute release kinetics. In general, solute release kinetics is controlled by either [118]–[120]:

- Matrix degradation;
- Solute diffusion;
- Matrix swelling.

Matrix degradation is the rate-controlling step in the delivery of hydrophobic drugs from the poly(lactic-co-glycolic)acid (PLGA) [121](Fig. 1.15a). PLGA copolymers, as well as other polyesters, can be synthesized in a broad range of molecular weights and processed into almost any size or shape. At the same time, it can encapsulate molecules of virtually any size. It can be dissolved in various common solvents including chlorinated solvents, tetrahydrofuran, acetone or ethyl acetate [122].

In the initial stage of exposure, the release kinetics is faster because the solute molecules at the surface erode to the surrounding media (Fig. 1.15a) [121]. The initial burst effect is possible at this stage and is related to the solute concentration and solute physico-chemical characteristics [121], [122]. In a later stage of exposure (Fig. 1.15a), water hydrolyses the PLGA units into soluble oligomeric species and monomeric products [121], [122]. As degradation of the matrix proceeds, a passage for drug molecules to be released to the medium is created [122]. Thus, the release of the solute in this phase will mainly depend

on the degradation kinetics of the matrix. Factors to be considered regarding degradation kinetics of a PLGA copolymer are:

- Drug role in attracting the aqueous phase into the matrix [122]. For example, aspirin causes enhanced swelling of the PLGA discs and accelerates their degradation. On the other hand, haloperidol hinders water absorption which causes prolonged degradation [123].
- Dimensions of PLGA carrier system due to the heterogeneous process of hydrolytic degradation [124], [125]. It was reported that thick PLGA coatings are degraded faster than thin PLGA coatings¹²⁴.
- Optimization of the polarity and molecular weight of the copolymer matrix [122]. PLGA consists of the more-hydrophilic polyglycolic acid (PGA) units and more-hydrophobic polylactic acid (PLA) units. Increase in the lactide content prolongs the degradation of PLGA matrix and results in the more sustained release of hydrophobic drugs (Fig. 1.15b). Similarly, PLGA containing copolymer units with a larger molecular weight degrades slower [122].

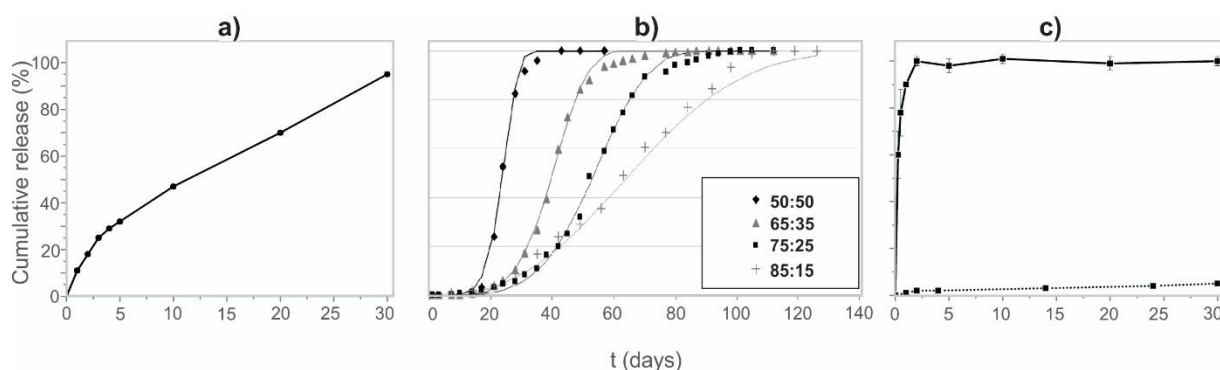


Figure 1.15: a) The release kinetics of estradiol from PLGA matrix, adapted from [121]; b) Model study of zero-order release kinetics of hydrophobic solute from PLGA matrix when lactide : glycolide ratio is varied [122]; c) The release kinetics of nalbuphine hydrochloride salt (black solid line) and nalbuphine decanoate (black dotted line) from PLGA matrix, adapted from [126].

In contrast, the delivery of hydrophilic drugs from PLGA is solute diffusion controlled. The hydrophilic formulation of the drug (nalbuphine hydrochloride salt) is released completely from the PLGA matrix in the first two days of exposure (Fig. 1.15c) [126]. Thus, drug molecules completely diffused out to the medium before the matrix degradation was able to influence their release. In contrast, just 5 % of the hydrophobic drug formulation (nalbuphine decanoate) was released from the PLGA matrix within 30 days of exposure (Fig. 1.15c) [126].

Matrix swelling is commonly observed for chitosan-based drug carriers as chitosan contains abundant amine groups within polymer chains. It is a biocompatible, biodegradable and nontoxic material [127]. When such systems come into contact with water, relaxation of polymer chains takes place. The dissolved drug diffuses into the external medium, crossing the swollen polymeric layer formed around the matrix. As a result, the drug is usually completely delivered within one day of exposure [128].

The design of polymer-based systems for dissolution control of inorganic particles (V_2O_5 , MgO) has to consider the particulate nature of the solute. Our recent study showed that the application of 1D nanowires instead of sub-micro isotropic V_2O_5 particles proved to be

effective against initial burst release of the vanadium pentoxide nanowires. The V₂O₅/polymer interactions proved to be equally important in the control of vanadate release toward the media. Because of that, PLGA with monomer ratio 50:50 is more effective in the prevention of an initial burst effect than either PLGA with monomer ratio 85:15 or PLA. The underlying mechanism includes interfacial interactions which stabilize PLGA nanowires at the surface even though they are partially exposed to the external media. Because of that, they do not undergo dissolution via the radial wire breakage mechanism. The chitosan proved to be an inappropriate carrier for the V₂O₅ because its swelling highly stimulates its dissolution and delivery of vanadate ions [100].

To summarize, according to the presented literature we came to the conclusion that the stabilization of either V₂O₅ or MgO within polymer-based coatings requires addressing the impacts of (i) **polymer matrix characteristic** (polarity, molecular weight, coating thickness and swelling), (ii) **particle morphology** (isotropic or 1D anisotropic particles) and (iii) **the dissolution mechanism** of inorganic nanoparticles on the delivery kinetics of either vanadate or magnesium ions. These factors were starting points of the research on designing effective and safe coatings able to combine antibacterial and bioactive activities of MgO and V₂O₅ which will be presented in this doctoral dissertation.

Chapter 2

Aims and Hypothesis

2.1 Aims

The main aim of the dissertation was to design innovative:

- Magnesium oxide-based multifunctional biocoatings able to prevent bacteria attachment to the biocoatings surface and exhibit a sustained release of magnesium towards the medium.
- Vanadium pentoxide-based multifunctional biocoatings able to prevent bacteria attachment to the biocoatings surface and exhibit a controlled delivery of vanadates within the desired bioactive concentration range (2.5 to 10 μM).

In this way, I strived to develop medically applicable coating which joins together the ability to protect the surface of the implant from attachment of bacteria and formation of biofilms with the possibility to stimulate regeneration and implant's postoperative integration by the means of sustained release of metal ions within bioactive concentration limit.

For that purpose, the doctoral thesis will include:

- The optimization of vanadium pentoxide structure and morphology regarding antimicrobial activity on the basis of myeloperoxidase-like catalytic activity;
- The optimization of magnesium oxide structural and morphological characteristics regarding its contact-based antimicrobial activity;
- The synthesis and in-vitro characterization (prevention of surface-biofilm formation, control of vanadate/magnesium ions release kinetics) of the multifunctional V_2O_5 /polymer and MgO /polymer composite coatings.

My work strived for answers to the following questions:

- How fundamental principles of particle growth and nucleation could be utilized to obtain different morphologies and structures of MgO and V_2O_5 ?
- Is it possible to maximize antibacterial potential of metal-oxides by tailoring their structural and morphological characteristics?
- How the interphase interactions, particle morphology and agglomeration influence stabilization of metal-oxide particles within polymer matrix in *in-vitro* conditions (V_2O_5 against dissolution and MgO against hydration)?

- What are the mechanisms of interactions of the designed coatings with both, bacteria and human cells?

2.2 Hypothesis

I proposed that tailoring of metal-oxide structural and morphological properties, reduction of particle agglomeration and regulation of metal-oxide/polymer-matrix interphase interactions would enable coupling of bioactive and antibacterial properties of either vanadium pentoxide or magnesium oxide within polymer-based composite coatings.

The embedding of 1D metal-oxide nanoparticles within polymer matrixes could be a solution. I believe that anisotropy of particles could help hinder their diffusion and erosion from the matrix towards the media, and thus allow their efficient stabilization within the matrix. Because of that, particles (solid form) would stay on the surface and their antimicrobial activity would be retained. The experimental evaluation of underlying principles related to solubility control of either V_2O_5 or MgO (and controlled release of either vanadate or magnesium ions, respectively) included application of following polymers:

- Chitosan;
- PLA;
- Poly(lactic-co-glycolic)acid with monomer ratio 50:50 (PLGA50);
- Polycaprolactone (PCL);

I hypothesized that incorporation of nano-textured MgO microrods within polymer-based biocomposites will be efficient way of improving their contact-based antibacterial activity. The starting point included MgO microrods' nano-textured surface with optimized quantity of surface defects which would exhibit higher stability (less agglomeration) and stronger bactericidal potential in comparison with the MgO nanoparticles.

The hypothesis regarding coupling of V_2O_5 myeloperoxidase-like catalytic activity with its controlled dissolution was based on incorporation of V_2O_5 inside polymer-based biocomposites. The starting point included tailoring the V_2O_5 structure (intercalation of various dopants) and providing 1D morphology (nanowires, nanoribbons, nanorods, etc.) to obtain superior characteristics compared to commercial isotropic V_2O_5 particles.

Chapter 3

Materials and Methods

3.1 Materials

The following materials were used for synthesis of inorganic particle/polymer matrix composite coatings:

- Magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, Fluka, 99 %);
- Ammonium carbonate ($(\text{NH}_4)_2\text{CO}_3$, Alfa Aesar, 99.5 %);
- Nitrogen gas (N_2 , Messer, 5.0);
- Magnesium oxide nanoparticles (MgO , Alfa Aesar, 99.5%, APS powder);
- Deionized water (resistivity below $18\mu\Omega \cdot \text{cm}$);
- Hydrogen peroxide (H_2O_2 , 30 % solution, Carbo Erba Reagents, ACS reagent);
- Vanadium pentoxide (V_2O_5 , Alfa Aesar, 99.8 %);
- Ammonium metavanadate (NH_4VO_3 , Acros Organics, ACS reagent);
- Potassium hydroxide (KOH , Sigma Aldrich, ACS reagent)
- Liquid nitrogen (N_2 , Messer);
- Sulfuric acid (H_2SO_4 , Sigma Aldrich, ACS reagent)
- Poly(lactic-co-glycolic)acid, monomer ratio 50:50, molar mass 60 000, (PLGA, Evonik);
- Polylactic acid, molar mass 380 000, (PLA, Evonik);
- Polycaprolactone, molar mass 100 000 (PCL, Sigma Aldrich, analytical grade reagent),
- Chitosan, molar mass 100 000 (Sigma Aldrich, analytical grade reagent);
- Polystyrene, molar mass 100 000 (PS, Sigma Aldrich, analytical grade reagent);
- Dichloromethane (DCM, Sigma Aldrich, analytical grade reagent);
- Ethanol (Sigma Aldrich, analytical grade reagent);
- Potassium bromide (KBrO_3 , Sigma Aldrich, ACS reagent).

The following materials were used to characterize *in-vitro* release kinetics and morphology changes of the composites:

- Phosphate buffered saline (PBS, in the form of tablets, Sigma Aldrich 99 %);
- Sodium monobasic phosphate (Na_2HPO_4 , Sigma Aldrich 99 %);
- Sodium dibasic phosphate dehydrate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, Sigma Aldrich, ACS reagent);

- Sodium Chloride (NaCl, Carlo Erba Chemicals, ACS reagent);
- Mueller-Hinton broth (MH, Fluka);
- Luria-Bertani broth (LB, Fluka);
- Foetal bovine serum (FBS, Sigma Aldrich, sterile filtered);
- McCoy's 5A modified medium (Sigma Aldrich, powder).

The interactions of bacteria and cells were measured by an application of following reagents:

- Agar (Fluka, for microbiology);
- D-glucose(Life Sciences, for biotechnology);
- Crystal violet (Merck, ACS reagent);
- Resazurin (Sigma Aldrich, >93 %);
- *BacLight Live/Dead* assay (Omega, L7007);
- Glutaraldehyde (25 % solution, Sigma Aldrich, grade I);
- Osmium tetroxide (Sigma Aldrich, sealed ampule);
- Sodium citrate (Sigma Aldrich, BioUltra reagent).

3.2 Synthesis Methods

3.2.1 Synthesis of nano-textured MgO microrods

The modified procedure of Dhal *et al.* was used to prepare the precursor in the form of microrods [42], as illustrated in Fig. 3.1. The stoichiometric amounts of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and $(\text{NH}_4)_2\text{CO}_3$ were separately dissolved in 100 ml of deionized water, each. As-prepared solutions were mixed and subjected to vigorous stirring (1000 rpm) for 6 h. The obtained precipitate was washed three times with deionized water and dried overnight in ambient conditions under a hood.

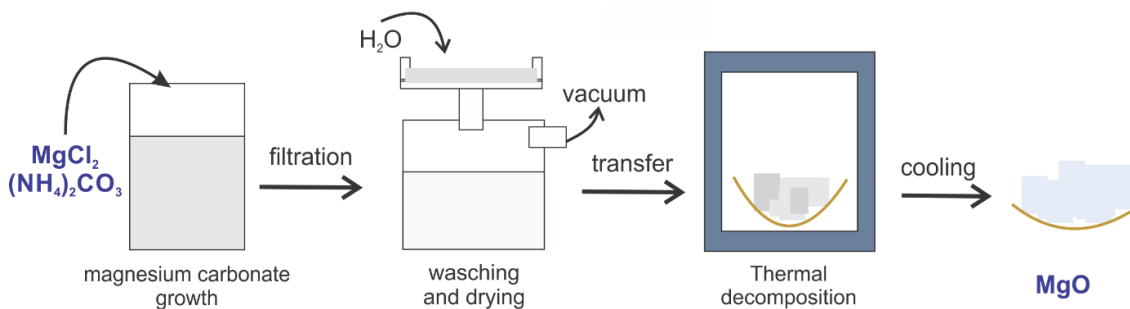


Figure 3.1: Schematic representation of two-step method for synthesis of MgO microrods.

A total of 200 mg of the dried precursor was thermally decomposed either in air or in pure N_2 in a two-step heating cycle. In the first step, in 90 min the precursor was heated to the calcination temperature (T_{calc}), which was varied from 400°C to 1000°C . In the second step, the precursor was maintained for 3 h in the particular calcination conditions. After the end of the heating cycle, the powders were cooled naturally inside the oven and saved for physicochemical, antimicrobial and hemocompatibility characterizations. The preparation conditions and abbreviations of all the synthesized MgO microrods are summarized in the Table 3.1. Their properties were compared to the commercially obtained MgO nanoparticles (MgO NPs, obtained by the means of unknown synthesis procedure).

To obtain a more representative control, I subjected them to thermal annealing in air at the previously described conditions for thermal decomposition of the precursor. As-processed powder is abbreviated as AN MgO NPs. The obtained particles were stored in a desiccator under vacuum for further characterization of physicochemical properties, antibacterial activity and hemocompatibility.

Table 3.1: The processing conditions of the prepared MgO powders.

abbreviation	MgO 400	MgO 600	MgO 700	MgO 800	MgO 900	MgO 1000	MgO 900 N2
T _{calc}	400	600	700	800	900	1000	900
atmosphere	air	air	air	air	air	air	N ₂

3.2.2 Synthesis of nanostructured vanadium pentoxide

The vanadium pentoxide nanostructures were synthesized hydrothermally. The 1D nanostructured V₂O₅ was synthesized using a hydrothermal method [85]. Commercial V₂O₅ powder was dissolved in 50 ml of aqueous solution of H₂O₂. The final vanadium concentration was 0.06 M and the molar ratio V:H₂O₂ was 1:10. After 30 min, a yellow-orange peroxovanadate solution was obtained and transferred to a Teflon-lined autoclave. It was sealed and held at 200°C for a 24h hydrothermal treatment. At the end of the process the precipitate was collected, washed with deionized water and redispersed in deionized water. The suspensions were fast-frozen in liquid nitrogen and lyophilized at -100°C (Fig. 3.2a).

The preparation of 1D vanadium pentoxide xerogel particles was performed from acidified vanadium metavanadate solution [88]. The ammonium metavanadate was dissolved in 50 ml, so that final concentration of the precursor was 0.1 M. It was acidified by 1M sulfuric acid to the set pH value to 1.7. As-obtained precursor solution was transferred to a Teflon-lined autoclave, sealed and subjected to a 24h hydrothermal treatment at 240°C. At the end of the process, the precipitate was treated the same way as the vanadium pentoxide nanowires (Fig. 3.2b).

The intercalation of potassium was performed according to the literature [89]. The commercial V₂O₅ powder was suspended in 50 ml deionized water with the final concentration of 0.1 M. The 1M solution of KOH was added to obtain a molar ratio of V:K was equal to 3:1. The mixture was vigorously stirred for 24 h at room temperature before it was transferred to a Teflon-lined autoclave. It was sealed and held at 220°C for a 24h hydrothermal treatment. At the end of the process, the precipitate was treated the same way as the vanadium pentoxide nanowires (Fig. 3.2c).

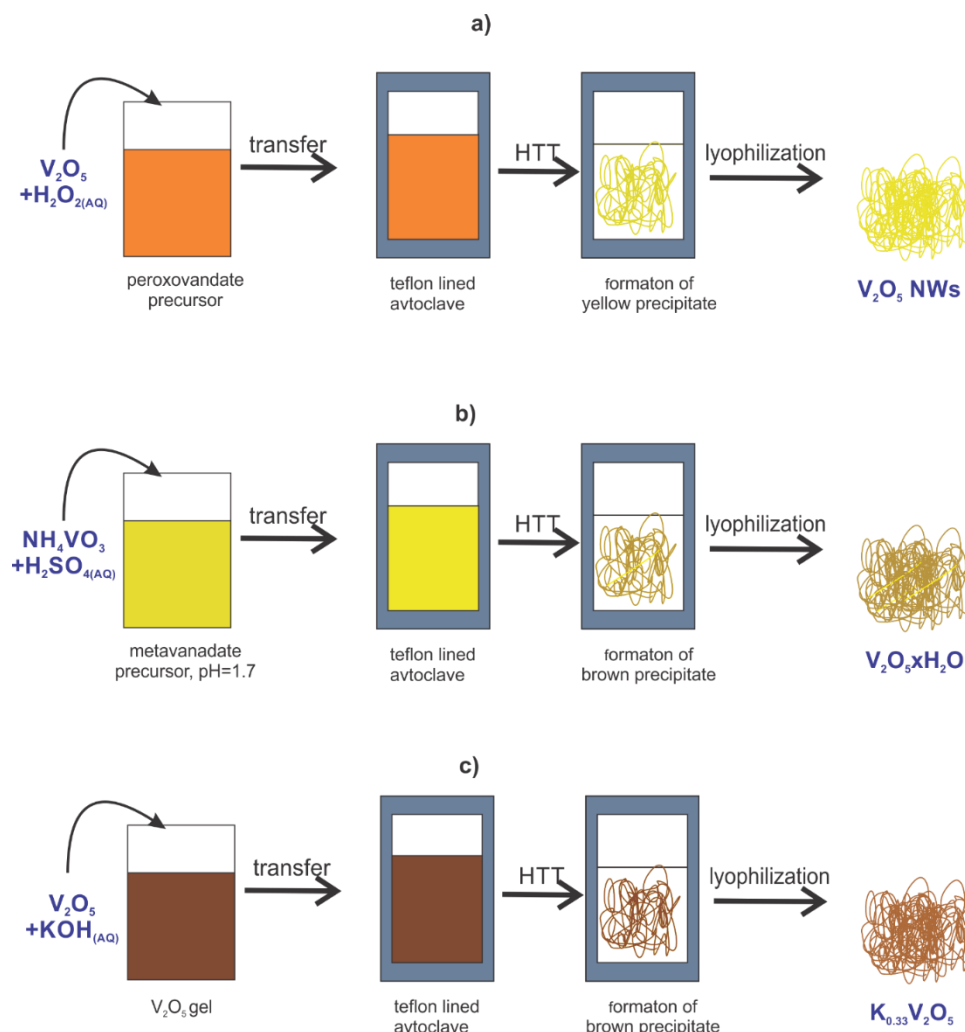


Figure 3.2: Schematic representation of the used hydrothermal methods for synthesis of the a) V_2O_5 NWs, b) $V_2O_5 \cdot xH_2O$ and c) $K_{0.33}V_2O_5$.

3.2.3 Synthesis of MgO/polymer and V_2O_5 /polymer composite coatings

A solvent-casting method was used to prepare the composite coatings. The total mass of the composite coatings was set to 60 mg. The solution (40 mg/ml) of either PLGA50, PS or PLA in dichloromethane (DCM) was mixed with 1.5 ml of a suspension of V_2O_5 (varied from 0.08 to 4 mg/ml) in dichloromethane and left under the hood overnight to allow for the solvent's evaporation. The incorporation of MgO within the MgO/polymer composites followed the same procedure.

Chitosan (purity 99 %, Sigma Aldrich) is soluble in an acidic aqueous solution and therefore a 1 % (v/v) acetic acid solution was used instead of dichloromethane throughout the composite coating's preparation procedure. The preparation of V_2O_5 /PS composites followed the same processing procedure as for V_2O_5 /PLA composites.

3.3 Characterization Methods

3.3.1 Methods of physico-chemical characterization

3.3.1.1 X-ray diffraction (XRD) analysis

The phase composition of the prepared inorganic materials (either MgO or V₂O₅) was analysed using a Bruker AXS D4 Endeavor X-ray diffractometer in the angular range from 5° to 70° with a 3 s step of 0.02°. For this purpose, as-prepared nanostructured vanadium pentoxide materials (without previous crushing in a mortar) were placed on the XRD holder and sealed with Kapton foil. Data were analysed using XPert Highscore software.

The crystallinity of the studied MgO powders, expressed as an average crystallite size (d), was determined by the means of using Scherrer equation based on the full-width half maximum (β , FWHM) of (002) absorption maximums, according to Eq. (3.1).

$$d = \frac{0.9 \cdot \lambda}{\beta \cdot \cos(\theta)} \quad (3.1)$$

where θ represents the Bragg angle. I assumed a round shape of the crystallites.

The conversion factor of oxide-to-hydroxide transformation (x) for the duration of MgO exposure to various physiological and growth media, according to Eq. 3.2.

$$x = \frac{A_{\text{hydroxide}}^{(101)}}{A_{\text{hydroxide}}^{(101)} + A_{\text{oxide}}^{(200)}} \quad (3.1)$$

where $A_{\text{hydroxide}}^{(101)}$ represents the area under the Bragg diffraction peak of Mg(OH)₂ (101) planes while $A_{\text{oxide}}^{(200)}$ represents the area under the Bragg diffraction peak of MgO (200) plane. These areas were calculated using the “XPert Highscore” software.

3.3.1.2 Scanning and transmission electron microscopy (SEM and TEM)

The examination of the morphology of the either inorganic particles or the composites surface was performed by the means of SEM analysis (JEOL JSM 7600F). The particles were dispersed in ethanol and dried on an Au-sputtered, 100-nm polycarbonate filter membrane (IsoporeTM Polycarbonate Membrane Filter, Sigma Aldrich). The membrane was fixed on the carbon tape and coated with approximately 5 nm of carbon. The inorganic-particle/polymer composites were stuck on the carbon tape and carbon coated before observation. High-resolution TEM and selected-area diffraction (SAED) analyses were performed on a JEOL JSM 2100 transmission electron microscope with a LaB6 electron source in order to evaluate the crystal structure of the prepared inorganic nanoparticles. Before the observation the particles were ultrasonically dispersed in ethanol. The as-prepared suspension was dripped onto a Cu TEM grid covered with lacey carbon.

3.3.1.3 Specific-surface-area analysis

The specific surface areas of the inorganic powders were determined with the Brunauer-Emmett-Teller (BET) method using a Micromeritics Gemini II 2370 nitrogen-adsorption apparatus (Norcross, GA).

3.3.1.4 Thermogravimetric analysis

The desorption thermodynamics of the MgO surface adsorbates was evaluated using thermogravimetric analysis (TGA, Netzsch STA 449 C/6/G Jupiter) coupled with mass spectrometry (MS, Netzsch QMS 403). Samples (5 to 10 mg) were heated up to 900°C with a rate of 10°C/min in argon. *In-situ* change of mass was recorded. The evolved gaseous phase was transferred to MS to elucidate its chemical composition. Before the measurement, powders were exposed to ambient conditions for 7 days.

The amount of both, the surface bonded and the intercalated, water in the vanadium pentoxide nanostructures was determined using TGA. Similar as in the case of MgO, 5-10 mg of a powder of interest was heated up to 500°C with a rate of 10°C/min in argon. *In-situ* change of mass was recorded and the evolved water vapour was transferred to MS in order to assess the samples regarding the presence of other impurities.

3.3.1.5 Fourier-transform infrared spectroscopy (FTIR)

The nature of water bonding to the surface of MgO was studied by the means of FT-IR analyses. It was conducted on a Perkin Elmer Spectrum 400 MIR spectrophotometer using either the attenuated total reflection (ATR) or the diffusion-reflection (DRIFT) technique. In the ATR mode, 3 mg of powder was placed above a diamond crystal and pressed. In the DRIFT mode, 5 mg of MgO powder was mixed with 70 mg of KBr and pressed into the tablet form. The measurement was performed relative to the KBr reference. The ATR-IR spectra were obtained in the range from 650 to 4000 cm⁻¹, while the DRIFT spectra were measured in the range from 450 to 4000 cm⁻¹. Before the measurement, powders were exposed to ambient conditions for 7 days to allow water vapour adsorption on the MgO surface.

The determination of inorganic-particle/polymer-matrix interactions interface included a collection of IR spectra relative to the polymer matrix. The mass ratio between the polymer and the V₂O₅ particles in the composites was 1:1 while it was 3:1 between MgO and polymer. The composite coating was detached from the glass bottom and cut into 10x10 mm piece. It was placed above the diamond crystal and pressed. The ATR-IR spectra were obtained in the range 650 to 4000 cm⁻¹. The positive control measurement involved a measurement of ATR-IR spectra of pure polymers without the metal-oxide phase.

3.3.1.6 UV/Vis reflectance

The electronic structure of MgO surface was probed by the means of the UV/vis reflectance. It was performed by means of a spectrometer (UV-3600, Shimadzu) with an integrating sphere. Five milligrams of the sample was placed on top of the BaSO₄ tablet and pressed to obtain a flat surface. The obtained reflectance data were processed by Kubelka-Munk transformation. To prevent influence of water and carbon dioxide adsorption at the surface of MgO, the measurements were performed after the ending of thermal processing.

3.3.1.7 Photoluminescence (PL)

Ten milligrams of a powder was pressed inside the well of a black 96-well and transferred to the microplate reader (Synergy H1, BioTek). It was excited on different wavelengths (280 and 370 nm) and corresponding emissions were recorded. Similar to UV/Vis reflectance, the measurements were performed after the ending of thermal processing.

3.3.2 *In-vitro* stability and ion release

3.3.2.1 Dissolution of inorganic particles

To determine the behaviour of the studied MgO particles in various aqueous environments, 30 mg of a material was placed in 15 ml of a medium (e.g. PBS, physiological solution, MH broth and FBS+MC broth) at 37°C and shaken at a rate of 60 rpm. After regular time intervals (0, 2h, 6h, 24 h, 96 h), the suspension was filtered over a 5 µm sized filter paper. The obtained precipitates were stored for further analysis of physico-chemical properties.

To determine the solubility kinetics of pure V₂O₅ particles, 5 mg of material was placed in 10 ml of phosphorus buffered solution (PBS) at 37°C and shaken at a rate of 60 rpm. The 500-µL aliquots were taken at regular time intervals and centrifuged in a MPW-55 micro-centrifuge at 14,500 rpm for 15 min to obtain clear, particle-free supernatants that were removed and stored for a later quantification of the vanadate ions. The measurements were performed in triplicate.

3.3.2.2 Release of the inorganic ions from the composites

The elution kinetics of either magnesium or vanadate ions from the inorganic-particle/polymer composites was determined when 60 mg of the composite was exposed to 20 ml of PBS at 37°C and shaken at a rate of 60 rpm. The 500-µL aliquots were taken at regular intervals and stored for subsequent quantification of the released vanadates. After the exposure, the composites were washed, dried and stored for SEM observations of their surfaces. The measurements were performed in quadruplicate.

3.3.2.3 Quantification of the released ions

The concentrations of the magnesium ions released during the elution-kinetics studies were determined spectrophotometrically using 4-(2-pyridylazo)-resorcinol (PAR) and 0.5 M TRIS buffer solution in deionized water[129]. The reagent solution was prepared by dissolving of 0.054 mg of PAR in 49 ml of deionized water. 1 ml of 1M KOH was added to enhance dissolution. 300 µl of the reagent solution was mixed with 200 µL of the 0.5 M TRIS buffer and 100 µL of sample. Deionized water was added to the total volume of 1ml. The aliquots were transferred to transparent microplates and absorbance was measured at 506 nm by the means of multiplate reader (Synergy H1). Standard solutions for a calibration curve were prepared for the concentration range from 0.025 mg/L to 4 mg/L using MgCl₂ · 6H₂O.

The concentrations of the vanadate ions released during the elution-kinetics studies were determined spectrophotometrically using (PAR) and hydrogen peroxide [130]. A total of 400 µL of 2.5·10⁻⁵ PAR solution was mixed with 100 µL of a supernatant solution, 100 µL of 1-M H₂SO₄ and 100 µL of 3 % H₂O₂ (w/w) water solution. The deionized water was added to the total volume of 1ml. The as-prepared solution was kept for 2 h at room temperature and the absorbance was measured at 542 nm using a Shimadzu UV-3600 spectrophotometer. Standard solutions for a calibration curve were prepared for the concentration range from 0.025 mg/L to 3 mg/L using NH₄VO₃ (99.9 %, Sigma Aldrich).

3.3.3 Interaction with biological systems

I evaluated the interaction of the prepared materials with the bacteria and red blood cells (RBCs). The used bacterial strains were *E. coli* (ATCC 47076) and *S. epidermidis* (NCIMB 8853).

3.3.3.1 Micro dilution methods

I used the micro-dilution tests to obtain minimal bactericidal concentrations (MBC) of different MgO powders. They were ultrasonically dispersed in the MH broth medium and transferred to the 96-well plate with a flat bottom. The *E. coli* suspension in the MH broth medium was added to each well. The concentration of MgO was changing from 0.2 to 2 mg/ml, while the *E. coli* concentration was constant for all the samples (10^5 CFU/ml). Each composition was tested in four replicates. The plate was incubated at 37°C in a multiplate reader (Synergy H1, BioTek) and orbitally shaken at a constant rate of 250 rpm. The OD600 measurements were performed at regular time intervals. After 24 h of incubation, 50- μ l aliquots of the suspensions were smeared on agar plates, which were incubated overnight at 37°C. The lowest concentration, where the colony growth was not observed, was assigned as MBC.

3.3.3.2 Disc-diffusion test

A total of 100 mg of MgO powder was transferred to the 10-mm mould and pressed under 500 kPa to form a tablet. Overnight the culture of *E. coli* was centrifuged at 2000 g for 10 min to remove the medium solution. Bacteria were then re-suspended in a physiological solution (0.9 % NaCl), until the final concentration was 10^8 CFU/ml. 50 μ l of the *E. coli* suspension was uniformly smeared on a Mueller-Hinton (MH) agar plate to ensure the confluent growth of the bacteria. The MgO tablet was placed in the centre of the plate and incubated for 24 h at 37°C. After the incubation, the bacterial growth-inhibition zone was assessed.

3.3.3.3 Lethal potential of inorganic particles

I used the precise *live/dead* assay kit (L70007 BacLight, Molecular Probes) to monitor the lethal potential of the MgO powders against *E. coli*. The test was performed according to the user manuals [131]. Briefly, the ratio between the green- and red-light fluorescence is a linear function of the percentage of live bacteria, so the portion of live bacteria in a specific mixture is determined using the calibration curve, which is valid for the *E. coli* concentration of 10^8 CFU/ml [131]. The concentration of MgO was 1 mg/ml and each measurement was performed in four replicates. At regular time intervals, the ratios between the green- and red-light fluorescence were determined in the microplate reader and the percentages of the live bacteria were calculated. The kit allows the observation of the bacteria using fluorescence microscopy. One μ l of the bacterial suspension containing the *live/dead* assay was placed on an objective glass, covered with a cover glass and observed under a microscope with green- and red-fluorescent filters (Eclipse Ti-U inverted microscope, Nikon).

To determine the lethal potential of V_2O_5 against both, *E. coli* and *S. epidermidis*, 10^8 CFU/ml bacterial suspension was exposed to 0.2 mg/ml of the V_2O_5 . Before the exposure, the particles were dispersed using an ultrasound processor (Vibra Cell, VC 750) in a phosphate buffer. Each measurement was performed in four replicates. After the 8 h of bacteria' exposure to vanadium pentoxide, the suspension was diluted 10^5 times. The 50 μ l aliquot was transferred to the MH agar plate, smeared and incubated for 24 h. The number of grown colonies before and after the exposure to vanadium pentoxide was compared. As a negative control, bacterial suspension without vanadium pentoxide was used. The positive control was the bacterial solution in the 0.2 mg/ml solution of vanadate ions. During the experiment, the effect of added either chloride ions, hydrogen peroxide or both was evaluated.

3.3.3.4 SEM observation of bacteria

The morphology of the bacteria after 24 h of exposure to MgO in the MH broth was studied under the SEM. The bacteria grown in the MH broth were used as a negative control. The bacteria were fixed in 2 % glutaraldehyde in a 0.1-M phosphate buffer solution (pH = 7.4) at 4°C, post-fixed in a 0.5 % aqueous solution of osmium-tetroxide for 2 hours, dehydrated in 10 %, 30 %, 50 %, 70 %, 90 % and three times in 100 % ethanol solutions. Before drying, they were deposited on the polycarbonate membrane filters. The drying was performed using the critical-point drying method in a CPD030 dryer (Balzers). Before the SEM examination, the samples were coated with platinum in a sputter-coater SCD 050 (BAL-TEC).

3.3.3.5 ROS generation and antibacterial activity

A dihydrorhodamine 123 assay (DHR, >95 %, Sigma Aldrich) could be applied within a fluorescent assay for a general determination of the ROS species. It was dissolved in anhydrous dimethylsulfoxide (DMSO, Sigma Aldrich) in the Schlenk line under a nitrogen atmosphere to obtain the stock solution. It was stored at -30°C and protected from the light. Before the application the DHR was diluted in the physiological solution to obtain a working solution with a final concentration of 100 μ M. To measure the generation of ROS species the powders of interest were incubated with DHR in a physiological solution in two separate tests, with and without *E. coli*. The final concentrations were 1 mg ml⁻¹ (MgO), 10⁸ CFU ml⁻¹ (bacteria) and 10 μ M (DHR) [102]. During the incubation in the microplate reader at 37 °C and the continuous orbital mixing at 250 shakes per minute, the 490/530-nm fluorescence intensity was recorded every hour for 16 h. The measurements were performed in eight replicates.

The susceptibility of the *E. coli* to the MgO was measured in the MH broth medium by means of an MDT test in the presence of ascorbic acid, a ROS inhibitor. The mass concentration of the ascorbic acid throughout the test was 1 mg ml⁻¹; the concentration of MgO was varied from 0.5 to 1 mg ml⁻¹; and the bacteria concentration was equal to 10⁵ CFU ml⁻¹. As the used inhibitor is an acid, it reacts with the basic MgO which causes reduction in the concentration of the solid MgO. Because of that, the equimolar concentration of hydrochloric acid was employed as a negative control to account for the impact of the excessive particle dissolution on the antibacterial potential of the MgO in the presence of the ascorbic acid as a ROS inhibitor.

Electron spin resonance (EPR) spectroscopy was applied under ambient conditions using the Bruker ELEXSYS E500 spectrometer in a physiological solution. Spin trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO, Sigma Aldrich) with a final concentration of 25 μ M was used to trap the formed superoxide and hydroxide radicals. The final concentrations of MgO and bacteria in the microtube were 5 mg ml⁻¹ and 2 · 10⁸ CFU ml⁻¹, respectively. Before the EPR measurement the suspensions prepared in the physiological solution with DMPO were incubated at 37 °C and 250 rpm for the required time intervals. As a control, a 0.5 mM H₂O₂ solution was illuminated with UV A light (390 nm) for 2 min. The EPR spectra were measured immediately after illumination as well as after incubation at 37 °C and 250 rpm for the required time intervals. The instrument settings for the EPR measurements were: microwave power, 20 mW; microwave frequency, 9.315 GHz; modulation frequency, 100 kHz; modulation amplitude, 1 G; time constant, 40.96 ms; and sweep time, 40.96 ms. The measurements for each sample were made five times and the obtained data profiles were integrated to obtain the corresponding EPR spectrum.

3.3.3.6 Resazurin test

When bacteria are metabolically active, non-fluorescent resazurin is reduced to fluorescent resorufin [132]. Based on this property of resazurin, it was used to evaluate the metabolic activity of the *S. epidermidis*, which colonized either the PLGA or MgO/PLGA coatings' surfaces. The coatings were prepared inside 20 ml vials so that the concentration of MgO was 0.5 mg/cm². Some $2 \cdot 10^8$ CFU/cm² of bacteria in a sterile physiological solution were transferred to vials and incubated for 24 h. Measurements were performed in five replicates. The physiological solution was enriched with glucose, which led to biofilm formation on the coatings' surfaces instead of its formation at the liquid/air interface. After incubation, the coatings were washed with PBS. The 1 ml of resazurin solution was added to the vials and incubated for 30 min at 37°C. Aliquots (200 µL) were transferred to a 96-well microtiter plate and fluorescence at 530/590 nm was measured [132].

3.3.3.7 Crystal violet assay for testing biofilm formation

The *S. epidermidis* colonization of the V₂O₅/Polymer coatings' surfaces was assessed with the crystal violet (CV) assay. The coatings were prepared inside 20 ml vials so that the concentration of V₂O₅ was 0.1 mg/cm². Some 10^7 CFU/cm² of bacteria in a sterile physiological solution were transferred to vials and incubated for 24 h. Measurements were performed in five replicates. The physiological solution was enriched with glucose, which led to biofilm formation on the coatings' surfaces instead of its formation at the liquid/air interface. After incubation, the coatings were washed with PBS. The 1 ml of CV solution was added to the vials and left for 15 min to stain attached bacteria at the surface of coatings. In the next step, CV solution was removed and coatings were washed five times with deionized water. As prepared coatings were treated with 1 ml of Sorensen buffer for 30 min. Aliquots (200 µL) were transferred to a 96-well microtiter plate and absorbance at 540 nm was measured [133]. PLGA coatings without vanadium pentoxide were used as a positive control.

3.3.3.8 Live/dead test for detection of the viability of colonized bacteria

The BacLight makes it possible to observe the bacteria using fluorescence microscopy. The bacteria colonization of the coating's surfaces (either PLGA, MgO/PLGA or V₂O₅/PLGA) was performed as previously described. After rinsing with the PBS, the coatings were removed from the wells and the kit solution was added. The added volume was proportional to the measured CV absorbance. The coatings were then on an objective glass, covered with a cover glass and observed under the microscope with green and red fluorescent filters (Eclipse Ti-U inverted microscope, Nikon). The incubation time was fixed at 15 minutes.

3.3.3.9 Hemocompatibility tests

Fresh sterile whole sheep blood was supplied by the Institute of Microbiology and Parasitology (Veterinary Faculty, University of Ljubljana). The withdrawal of the blood was performed with sterile accessories from the healthy animals and was in accordance with the national guidelines (No. 510-05/13-3/2). Blood was stored in the Alsever's medium to be used within 7 days as a source of red blood cells (RBCs). The Alsever's medium contained 4.2 g/L of NaCl (Carlo Erba Chemicals, 99 %), 2.17 g/L of sodium citrate dihydrate (Sigma Aldrich, 99.8%) and 20 g/L of D-glucose (Sigma Aldrich, 99.5%). The experiments on the RBCs were performed in accordance with the national regulations (No. 003-02-5/2011-26) and biosafety guidelines implemented at the Jožef Stefan Institute.

Before the application, the RBC were diluted to the final concentration of the RBCs' of $5 \cdot 10^7$ cells/ml in the HEPES buffer. The coatings (either PLGA, MgO/PLGA or V_2O_5 /PLGA) were prepared as described before and exposed 4 ml of the RBC's suspension and incubated for 24 h at 37°C. During the incubation, the vials were linearly shaken in the water bath (Memmert) at the rate of 60 shakes per minute.

When the RBC membrane is damaged, the red-coloured haemoglobin is released into the surrounding media and absorbs light at 415 nm. The obtained absorbance is linearly proportional to the percentage of hemolysed cells [134]. After the incubation, 1 ml aliquots were taken and centrifuged at 1000 g for 10 min. The 200 μ L of obtained supernatants' were transferred to the microtitre plate to measure absorbance at 415 nm. The obtained absorbance was, from the calibration curve, used to quantify the percentage of hemolysed cells.

The morphology of the incubated RBCs, taken from the suspension above the coatings, was observed by the means optical microscopy (Eclipse Ti-U inverted microscope, Nikon). 1 μ L of the cell suspension was placed on the dry microscope objective glass, previously immersed in the 5 % bovine serum albumin, and covered with the cover glass. I was interested in the detection of the "ghost cells", i.e. the cells without the definite shape.

The morphology of RBCs, attached at the surface of the prepared coatings, was evaluated. For that purpose, after the incubation with RBCs, the coatings were detached from vial bottom and washed three times in physiological solution. In the next step, they were incubated for 30 min in the 2.5 % gluteral aldehyde in the physiological solution [134]. When the incubation was completed, the coatings were washed 3 times in water and transferred to the liquid nitrogen for fast freezing. After 5 min in the liquid nitrogen, the coatings were lyophilized. Before the SEM examination, the lyophilized coatings were placed on the sticky carbon tape and coated with approximately 5 nm of platinum in a sputter-coater SCD 050 (BAL-TEC).

3.3.4 Statistical analysis

From each set of measurement, the average values and sample standard deviations were calculated. Each sample population (at least 3 replicates) was tested for the existence of outliers. I used a two-sided *t*-test for independent samples to assess the differences between the two sample averages [135]. Throughout the dissertation, the presented error bars represent standard deviation values.

Chapter 4

Results

The MgO/polymer and V₂O₅/polymer coatings were formed by solvent casting of the metal-oxide suspension containing dissolved polymer. The main intentions were to couple the antibacterial activity of the metal-oxide nanostructured particles and their efficient dissolution control (e.g. sustained release of ions) within the polymer matrix.

The development of these innovative materials with advanced characteristics and high potential for improved biomedical application was performed in two steps. In the first step, design of the metal-oxide structural and dissolution characteristics for the purpose of maximizing antibacterial potential was performed. In the second step, physicochemical properties of the metal-oxide/polymer-matrix composites were tuned to couple sustained ion release and antibacterial properties against biofilm formation. At the end, hemocompatibility of the produced composite coatings was evaluated.

4.1 Nano-Texturing, Antibacterial Properties and Hydration of Magnesium Oxide Microrods

The nano-textured MgO microrods were prepared by the thermal decomposition of a magnesium carbonate precursor with the same morphology. During the process, the correlation between MgO microrods physicochemical properties, hydration kinetics and antibacterial properties was studied. The MgO NPs and An MgO NPs were used as reference materials.

4.1.1 Physicochemical properties of the nano-textured MgO microrods

The investigation of the MgO microrods nano-texturing included impact of the thermal decomposition conditions at the phase composition, MgO crystal structure, surface morphology and surface defects peculiarities.

The precursor, with a structure corresponding to the MgCO₃ · 2H₂O monoclinic crystal phase (Fig. 4.1a), was used to evaluate the nano-texturing of the MgO microrods. Its calcination ($T_{\text{calc}} = 400 - 1000^{\circ}\text{C}$) in air atmosphere caused a complete transformation to a cubic Fm3m lattice of MgO (JCSd card number 181456) (Fig. 4.1a). Thermal processing in nitrogen instead of air resulted also in the formation of MgO phase. The phase compositions of both, commercial MgO NPs and AN MgO NPs, were confirmed to be pure MgO (Fig. 4.1c). Thus, all the powders of interest exhibited pure MgO phase composition.

The diffraction maxima (e.g. for (200) crystallographic plane) were getting narrower with an increasing temperature (Fig. 4.1a), which indicated different crystallinity of the MgO powders. Because of that, the Scherrer's equation was used to calculate the average crystallite size. It increased from 3.8 nm (MgO 400) to 49.1 nm (MgO 1000) (Fig. 4.1b).

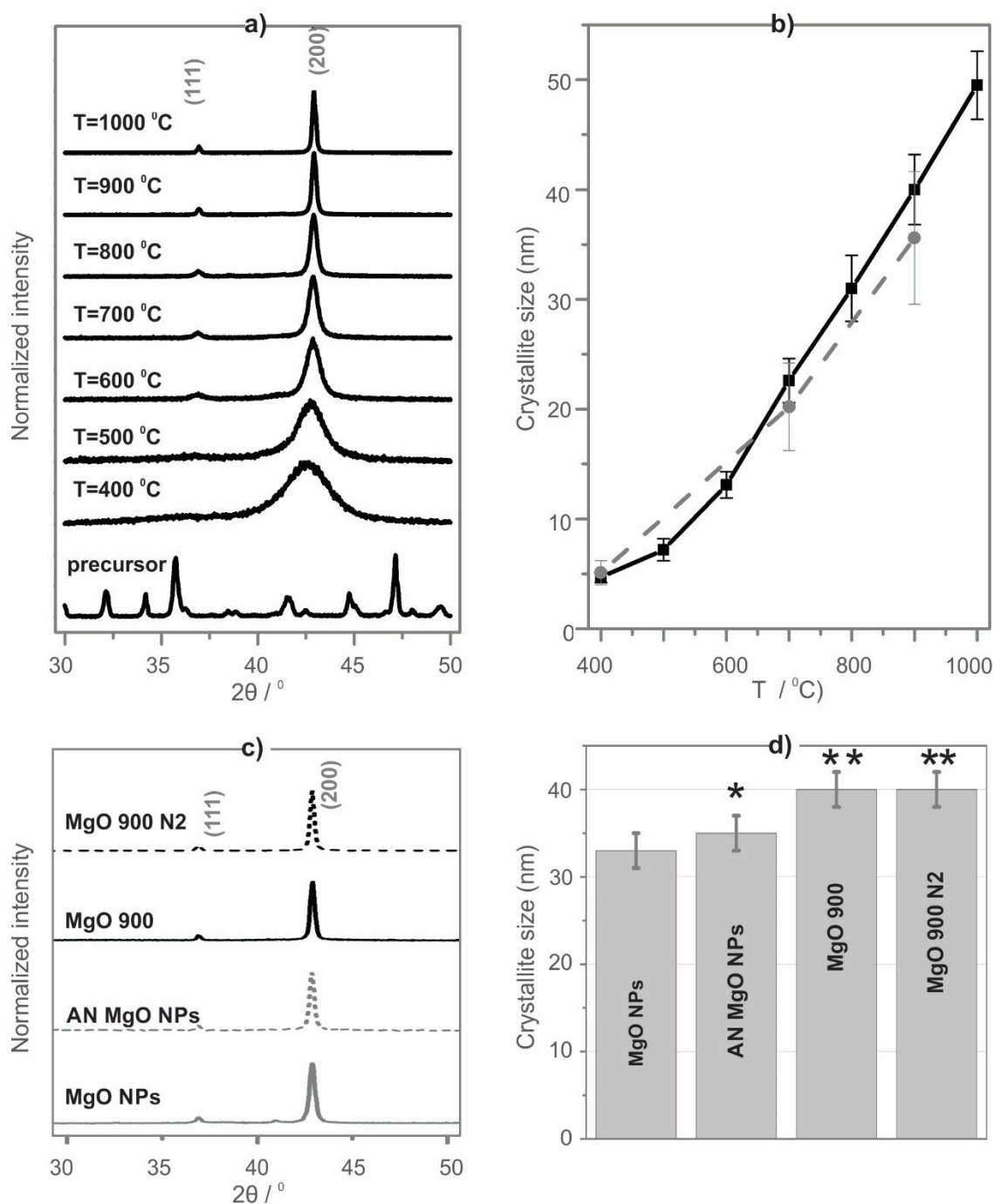


Figure 4.1: a) XRD patterns and b) average crystallite sizes calculated using the Scherrer equation (solid line) and measured on the TEM (dashed line) of MgO powders prepared under different calcination conditions; c) XRD patterns and d) average crystallite size calculated using the Scherrer equation of MgO powders subjected to either annealing or thermal processing in nitrogen.

The thermal decomposition of the precursor at 900°C in the pure N₂ did not cause change in average crystallite size, as calculated values for MgO 900 and MgO 900 N₂ were not statistically significant (41 nm vs 40 nm, Fig. 4.1d). In contrast, thermal annealing caused an increase in the Scherrer's diameter, from 33 nm (MgO NPs) to 36 nm (AN MgO NPs) (Fig. 4.1d).

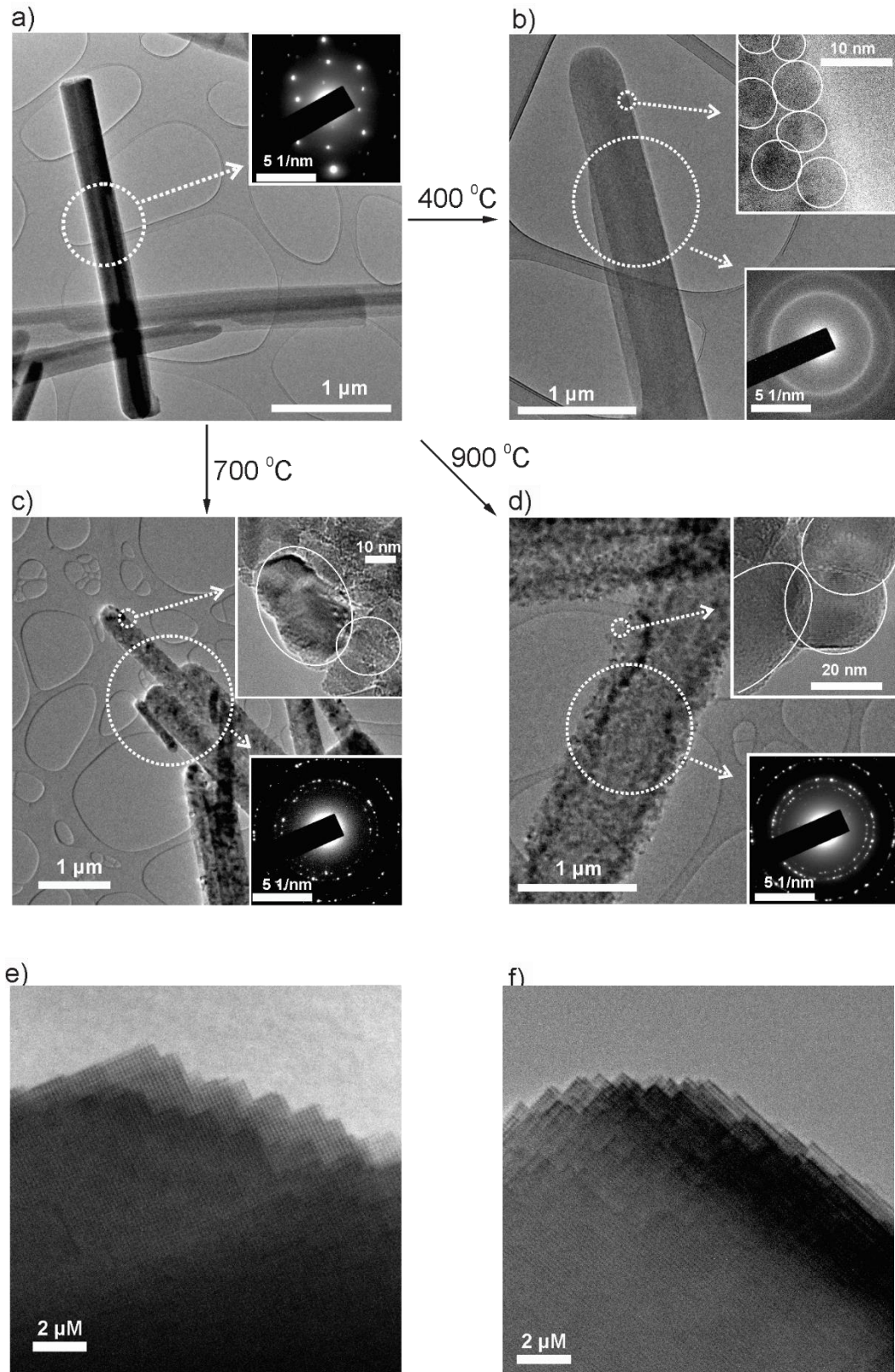


Figure 4.2: TEM and SAED investigation of the crystallographic structure of the a) precursor; b) MgO 400; c) MgO 700 and d) MgO 900 microrods; HR-TEM images of e) MgO 900 crystallite and f) MgO NPs nanoparticle. The MgO crystallites are labelled with white contours.

The calcination of MgCO_3 into MgO causes an almost three-fold decrease in the volume of material. Because of that, I studied the crystallographic and morphological changes of the particles during the process of thermal decomposition. The precursor particles exhibited a rod-shape morphology. The point diffraction pattern was observed when the SAED was collected from a single precursor rod (Fig. 4.2a), which demonstrated its single-crystalline nature. The calcination of precursor at 400°C resulted in polycrystalline rods as diffuse-ring diffraction pattern was observed by SAED (Fig. 4.2b). A closer examination revealed the rather smooth surface of the microrod, which was created from small MgO crystallites (Fig. 4.2b). In contrast, the microrods prepared at higher temperatures (700 and 900°C) led to polycrystalline microrods (SEAD patterns) with distinct features at the surface (Fig. 4.2c, d). A closer examination of the surface revealed surface texturing on the nano-scale, which was a consequence of a larger crystallite size in comparison with the MgO 400. These crystallites were irregular in shape, densely packed in the microrods and their impact on the MgO microrod surfaces' nano-texturing was progressively increasing with their size, i.e., with the thermal decomposition temperature. The semi-quantitatively determined average crystallite sizes were in good agreement with the average crystallite sizes determined by the means of the Scherrer equation (Fig. 4.1b).

The HR-TEM resolution image revealed the shape of MgO particles as “imperfect cubes”. The growth is consisted by the regular stacking of 100 planes along z -axis. These planes were not completely matching in area (x and y direction), causing the irregularities at the edges (Fig. 4.2e, f). They were smaller and more pronounced at the surface of a MgO NPs crystallite (Fig. 4.2f) than at the surface of a MgO 900 crystallite (Fig. 4.2e).

A TEM image represents an averaged 2D projection of a 3D object because the electron beam is passing through the sample. In contrast, SEM detectors are detecting either the backscattered electrons or emitted secondary electrons from the specimen the surface. Because of that, SEM is a more surface sensitive technique and was applied to obtain more detailed insight into the texturing of MgO microrods. The surface of the MgO 400 appeared smooth, with a low BET area (Fig. 4.3a). The first irregularities (initiations of the texturing) were observed on the surface of the MgO 600 (Fig. 4.3b). The MgO prepared at higher temperatures (700 – 900°C) displayed distinct nano-texturing of the surface, which was related to the gradual increase of the crystallite size obtained with an increase of T_{calc} . (Fig. 4.3c, d, e). Processing at even higher temperatures ($T_{\text{calc}} = 1000^\circ\text{C}$) resulted in the intergrowth of crystallites at the microrod surface (Fig. 4.3f). The emergence of nano-texturing on the MgO microrod surfaces caused a progressive increase in the BET surface area, from $1.8 \pm 0.4 \text{ m}^2/\text{g}$ ($T_{\text{calc}} = 400^\circ\text{C}$) to $7.5 \pm 0.6 \text{ m}^2/\text{g}$ ($T_{\text{calc}} = 700^\circ\text{C}$). A further increase in T_{calc} caused a decrease in the BET surface area, which is equal to $6.1 \pm 0.3 \text{ m}^2/\text{g}$ for the MgO 1000, due to the increase in the size of the nano-features on the MgO microrod surface. Even though the nano-texturing of the MgO microrod surface led to a significant increase in the specific surface area, these values are an order of magnitude lower than the BET surface area of the commercial MgO NPs, which is equal to $35 \pm 3 \text{ m}^2/\text{g}$.

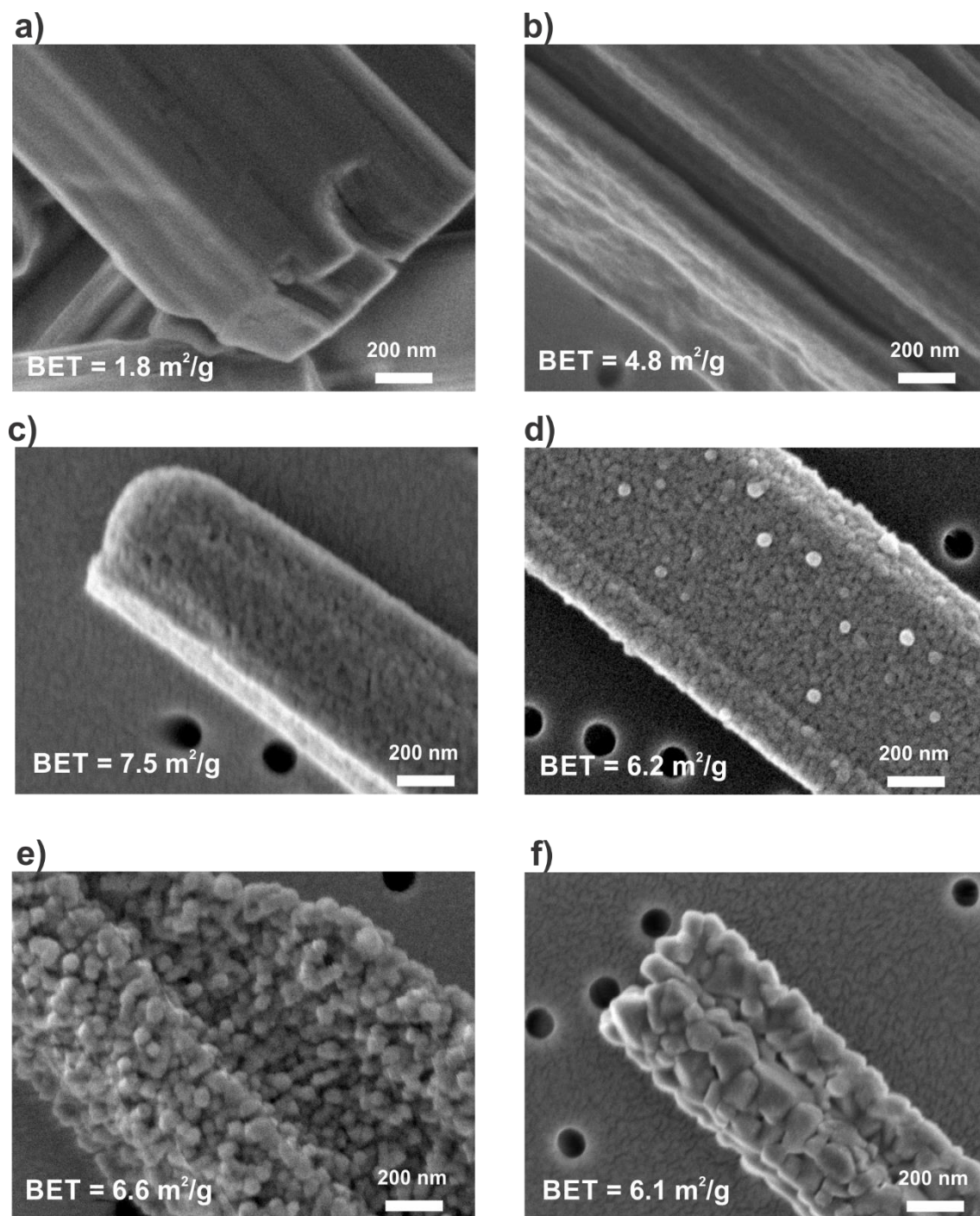


Figure 4.3: The SEM observation (50000x magnification) of nano-textured MgO microrods prepared at a) 400°C, b) 600°C, c) 700°C, d) 800°C, e) 900°C, f) 1000°C. The measured BET surface areas are provided in the lower-left-hand corner of the SEM images.

The nano-textured MgO 900 exhibited an average length of 16.7 μM (PI = 0.24) and average width of 0.48 μM (PI = 0.29), without any significant degree of particle agglomeration (Fig. 4.4a). In contrast, the commercial MgO NPs were intensively agglomerated (Fig. 4.4c), with agglomerates up to 4 μM . They exhibited a bimodal agglomerate size distribution with modes at 300 and 1300 nm. Neither thermal annealing nor processing atmosphere did not affect morphology of the MgOs. I did not observe any morphological differences between MgO 900 and MgO 900 N2, as they exhibited microrods

particle morphology with nano-textured surface (Fig. 4.4a, c) with no observable difference in average length and width. Similar to MgO NPs, AN MgO NPs powder consisted out of agglomerated nanoparticles where the agglomerate size was varying from 100 nm to 5 μm (Fig. 4.4d). In contrast to morphology, the specific surface areas were strongly influenced by the processing conditions (Fig. 4.3). Thermal annealing caused a decrease in BET area from 35 m^2/g (MgO NPs) to 22 m^2/g (AN MgO NPs). The atmosphere of thermal processing also exhibited strong impact, as BET increased from 6.6 m^2/g (MgO 900) to 14 m^2/g (MgO 900 N₂).

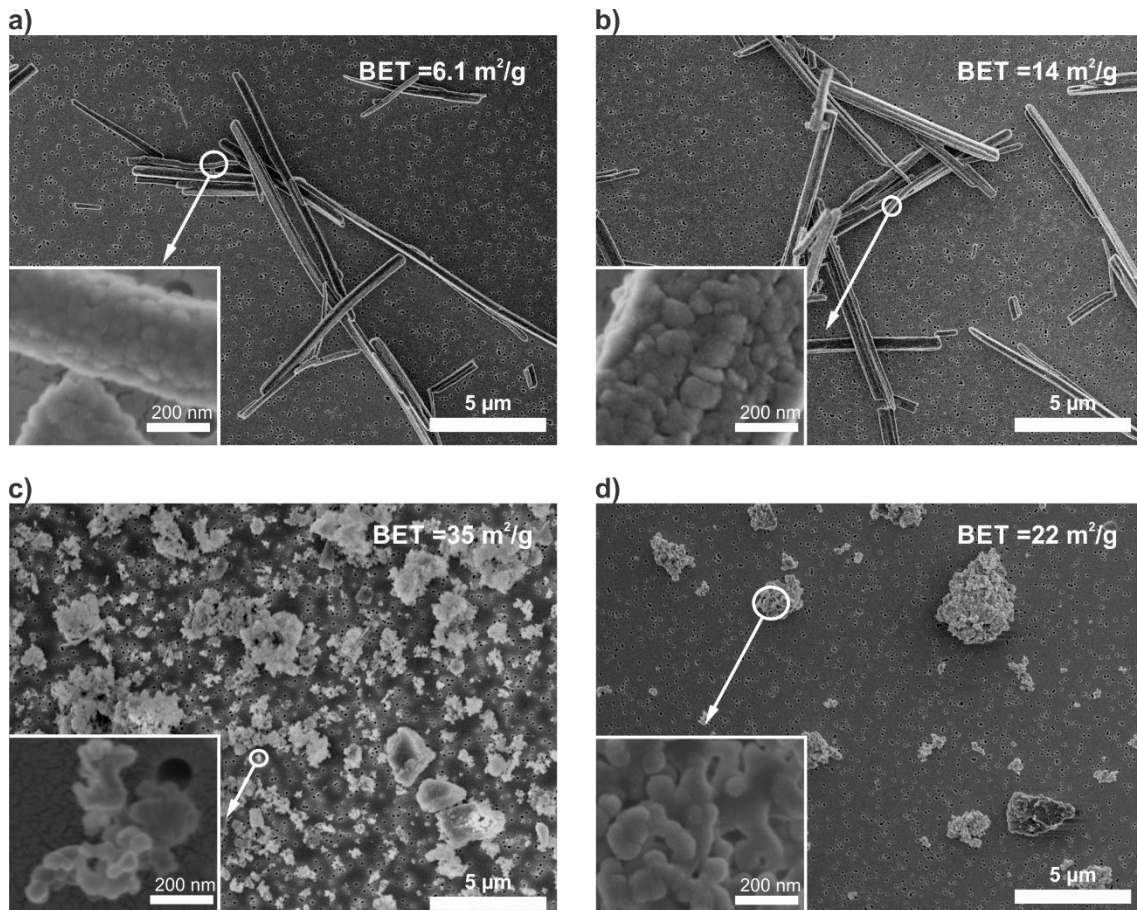


Figure 4.4: The SEM observation of a) MgO 900, b) MgO 900 N₂, c) MgO NPs and d) AN MgO NPs. The measured BET values are provided in the upper-right-hand corner of the SEM images.

The further investigation of the surface of the MgO powders was performed by the means of UV/Vis diffuse reflectance. Absorptions in the range 200–300 nm (6.2 to 4.1 eV) correspond to low coordinated oxygen atoms at the surface of the MgO [51]. The processing temperature of MgO had a significant impact on the UV reflectance spectra. The MgO 700 and MgO 900 microrods exhibited a low absorption in this range. In contrast, the smooth MgO 400 microrods absorbed at 260 nm, which indicated the presence of 3C atoms at their surface (Fig. 4.5a). Thus, we concluded that the calcination of MgO microrods at high temperatures (e.g., 700, 900°C) reduced the presence of low coordinated oxygen sites at the surface.

The commercial MgO NPs absorbed light strongly in near- and middle-UV region (Fig. 4.5b, grey line). It exhibited absorption maxima at 270 nm (4.6 eV, attributed to 3C-oxygen atoms) and 200 nm (6.2 eV, attributed to 5C oxygen atoms) as well as small hump

at 230 nm (5.4 eV, attributed to 4C oxygen atoms) [52], which indicated the presence of various types of the surface defects (e.g., kinks, corners, edges), i.e. a complex non-periodical surface structure (Fig. 4.5b).

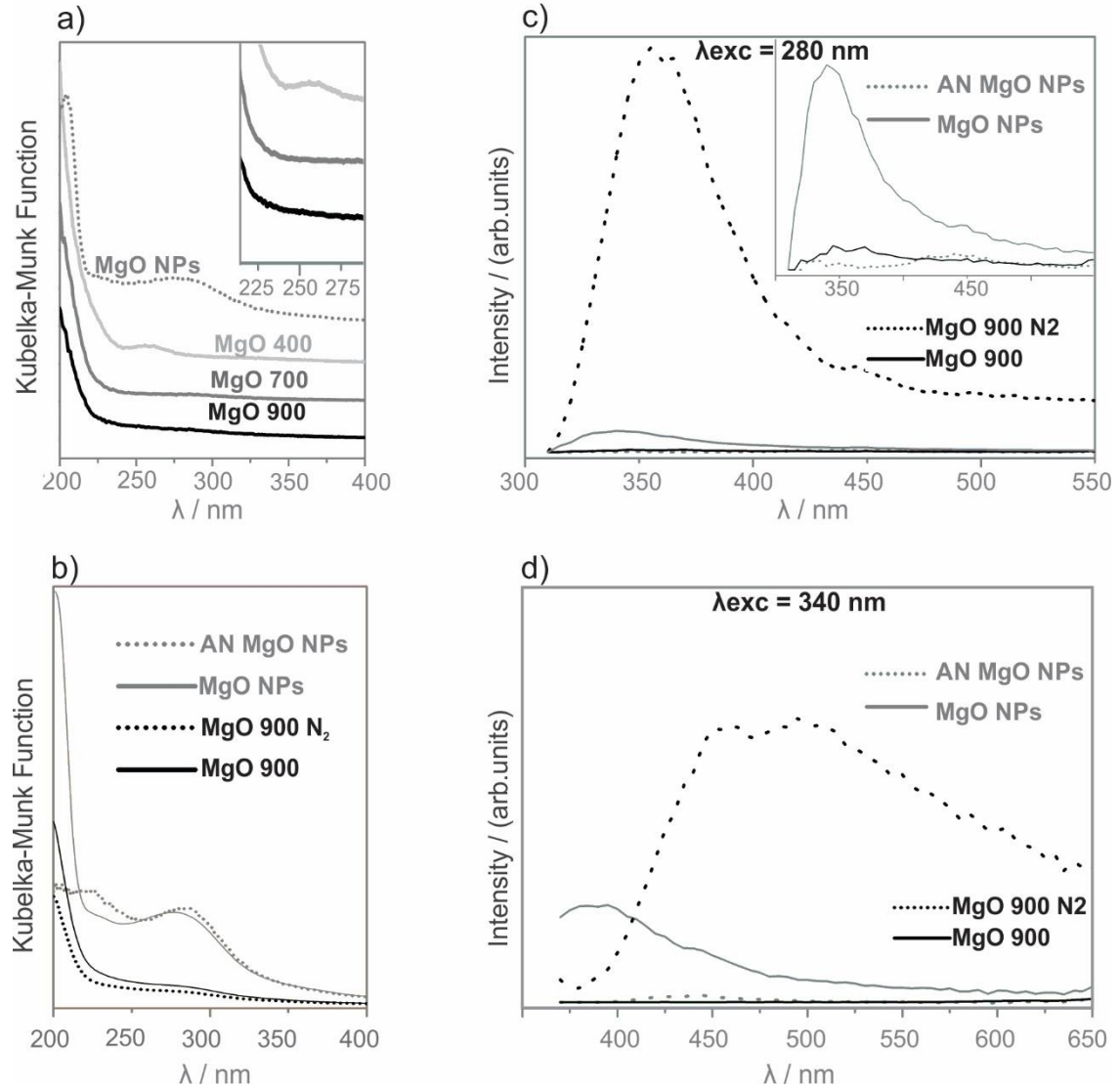


Figure 4.5: UV diffuse reflectance spectra of the studied MgO powders as a function of a) the thermal decomposition temperature and b) processing conditions (annealing and decomposition in nitrogen atmosphere). Photoluminescence spectra of the studied MgO powders at excitation wavelengths of c) 280 nm (the spectra of AN MgO NPs, MgO NPs and MgO 900 were zoomed-in) and d) 340 nm.

The thermal annealing in air atmosphere significantly altered the surface structure as AN MgO NPs UV reflectance (Fig. 4.5b, gray dotted line) exhibited i) the redshift of 3C oxygen absorption (280 nm, 4.4 eV), ii) the gain in relative intensity of 4C oxygen absorption (230 nm, 5.4 eV) and iii) the reduce of absorption intensity at 200 nm, related to 5C oxygen atoms. In contrast, MgO 900 (Fig. 4.5b, black line) and MgO 900 N₂ (Fig. 4.5b, dotted black line) exhibited lower light absorptions in a region from 200-400 nm, which directly indicated that abundance of LC oxygen atoms is lower than at the surface of MgO NPs and AN MgO NPs. The effect was more pronounced for MgO 900 N₂ than for MgO 900.

The PL emission spectra complements well with the UV reflectance spectra, as various defects can provide emissions of similar energy and not all the defects are emissive [136]. When excited at 280 nm (4.4 eV), the strong emission from MgO 900 N2 (Fig. 4.5c, dotted black line) was observed at 360 nm (3.4eV) and could be related to non-charged oxygen vacancies dimmers (F_2 defect site) [136]. MgO 900 (Fig. 4.5c, black line) and AN MgO NPs (Fig. 4.5c, dotted gray line) exhibited absence of light emission when excited at 280 nm. The MgO NPs exhibited emission maximum at 350 nm (3.5 eV) which I related, due to MgO hygroscopic properties, to isolated OH groups bonded at the surface [137]. It is important to state that PL emission spectra of MgO 900, MgO 900 N2 and AN MgO NPs were collected immediately after thermal treatment. Similarly, when excited at 340 nm, MgO NPs, exhibited broad emission spectra with maximum at 385 nm (3.2 eV), which might be attributed to bonded OH groups. In contrast to isolated OH groups' emission observed upon emission at 280 nm, these groups were forming hydrogen bonds [137]. The other three powders did not exhibit such peak. MgO 900 and AN MgO NPs did not exhibit any emission bands in this region while MgO 900 N2 exhibited broad emission band with maxima at 450 and 500 nm. These emissions might be related to isolated oxygen vacancies at the MgO surface (F and F^+ defect centres) and charged dimmers of oxygen vacancies (F_2^{2+} centers), respectively [136]. The maximal emission wavelength was higher than 385 nm (observed for MgO NPs) which might be due to difference in defect nature and abundance. To summarize, the PL study indicated that MgO 900 N2 was the only sample containing the detectable quantity of oxygen vacancies at the surface.

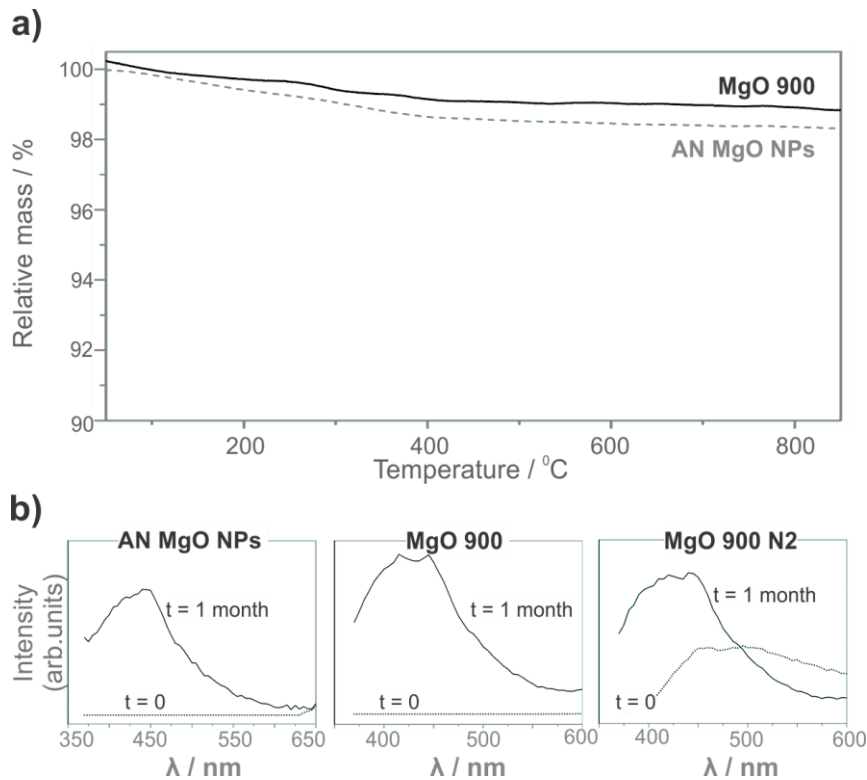


Figure 4.6: a) TGA determined mass loss profiles of either MgO 900 or AN MgO NPs immediately after the processing; b) evolution of the PL peaks due to water-vapor surface adsorption of MgO powders under ambiental condition.

I performed the measurement of UV reflectance and PL immediately after the preparation procedure ended. As a control, TGA spectra of the MgO 900 and AN MgO NPs were collected immediately after the treatment (Fig. 4.6a). In both cases the mass loss

during the measurement is below 1.5 %. In another set of control experiments, PL spectra were collected after 3 days and 1-month long exposure of powders to ambient conditions (Fig. 4.6b). After 3 days, just a slight emission of AN MgO NPs particles was observed. However, after one month powders started to emit light upon the excitation at 340 nm. Similar to MgO NPs, the emission spectra of AN MgO NPs, MgO 900 and MgO 900 N2 consisted of the broad maximum emerged. It could be related to the presence of absorbed impurities (i.e. OH included in hydrogen bonding). However, it exhibited two emission peaks (at 420 and 450 nm, Fig. 4.6b) in comparison to a single peak (385 nm, Fig. 4.5d) of MgO NPs. The emission peak related to oxygen vacancies, observed upon excitation of MgO 900 N2 disappeared (Fig. 4.6b). Such finding demonstrated reliability of photoluminescence measurements that were done immediately after thermal processing. It was also concluded that MgO NPs exhibited unique surface properties because the impurities' related emission maxima were different between MgO NPs and the other three MgO powders.

Further investigation of the MgO particles surface included IR spectroscopy after exposure of the powders (7 days) to ambient conditions. I was interested in absorption bands above 3200 cm^{-1} , which were attributed to both, physically absorbed and chemically bonded OH groups at the surface of MgO [73]. The MgO NPs exhibited broad maximum at 3300 cm^{-1} and sharp maximum at 3700 cm^{-1} (Fig. 4.7). The broad maxima might be related to vibrations of OH groups, which were involved in hydrogen bonding. In contrast, the sharp absorption was related to vibration isolated OH groups (not involved in OH hydrogen bonding) at the surface [73], [74]. Isolated OH bonds could be bonded at the LC atoms at the MgO surface (e.g. corners, kinks, step di-vacancies), while the hydrogen bonding OH groups are found on the terraces and edges [74]. The penetration depth of the IR ray does not depend on the wave number in the DRIFT mode. In contrast, in ATR mode of operation, the penetration depth (DP) depends on the wavenumber (W) according to Eq. 4.1 [138].

$$DP = \frac{1}{2\pi \cdot W \cdot n_C (\sin^2 \theta - n_{sc}^2)^{0.5}} \quad (4.1)$$

Because of that, the recorded signal would be inversely proportional to the wavenumber. For example, the ratio of intensities I_2/IR is equal to 0.4 in drift mode and only 0.15 in ATR mode. However, the ratio of intensities I_1/IR is close to one in both modes. This means that vibration maxima due to the isolated OH groups does not depend on the penetration depth, i.e. isolated OH groups were observed only at the MgO surface (Fig. 4.7a).

The AN MgO NPs, MgO 900 and MgO 900 N2 (Fig. 4.7b) exhibited just broad absorption maxima (3300 cm^{-1}) due to the hydrogen-bonding OH vibrations. The OH-MgO bond strength is varying and depends on the coordination of the bonding site at the MgO surface. Lower coordination of atoms at the surface means higher energy of bonding with OH groups.

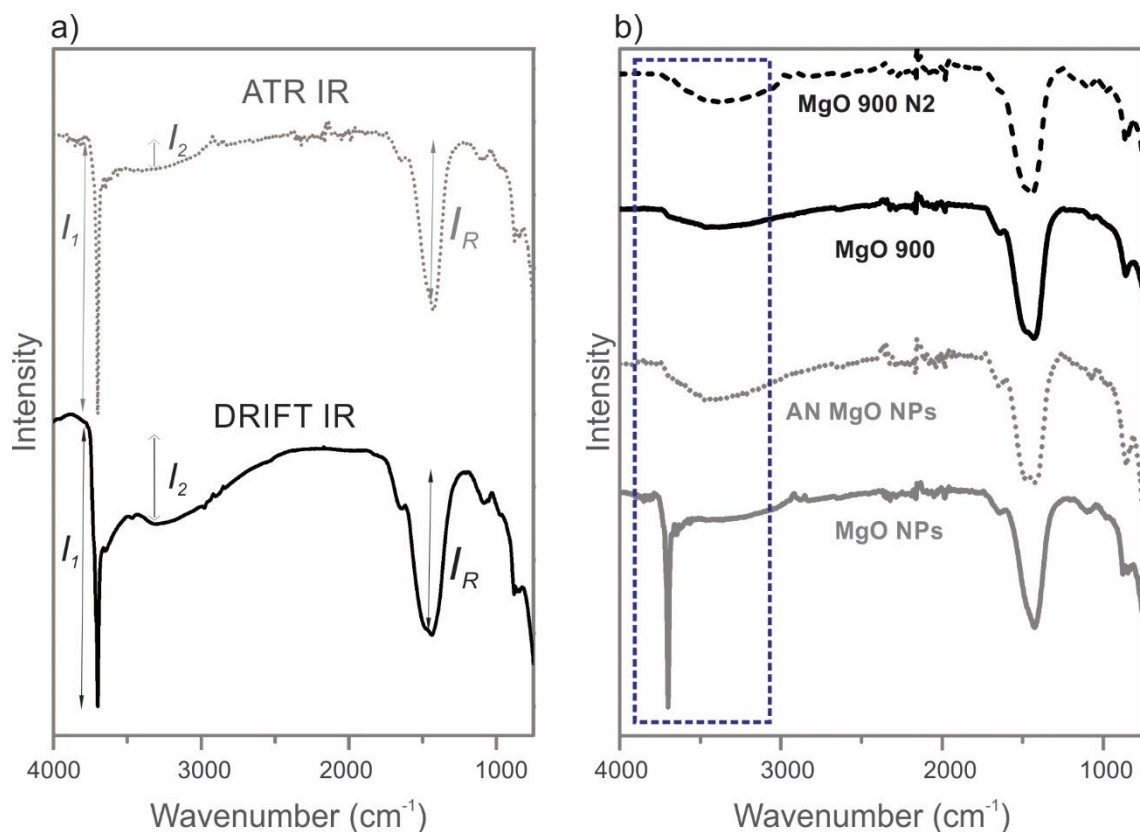


Figure 4.7: a) The IR spectra of MgO NPs in both, ATR and DRIFT modes; b) IR spectra of the studied MgO powders.

The simulation regarding thermal stability of OH groups reveals three domains. At temperatures up to 100°C, OH groups bonded at the terraces and edges should be removed. The OH groups placed at monoatomic steps and corners are removed up to 350°C, while the OH groups from kinks and step di-vacancies are removed at temperatures higher than 450°C [49]. Obtained experimental TGA profiles for the studied MgO powders (Fig. 4.8) showed that MgO NPs lost adsorbed impurities (water, CO_2) in three domains: i) up to 200°C, ii) from 250 to 350°C and iii) from 450 to 600°C. In contrast AN MgO NPs, MgO 900 and MgO 900 N2 lost adsorbed impurities within two temperature domains: i) up to 200°C and ii) from 250 to 400°C. These results (IR and TGA/MS) show that MgO NPs exhibit high abundance of kinks and step di-vacancies at the surface when compared to the other three studied powders.

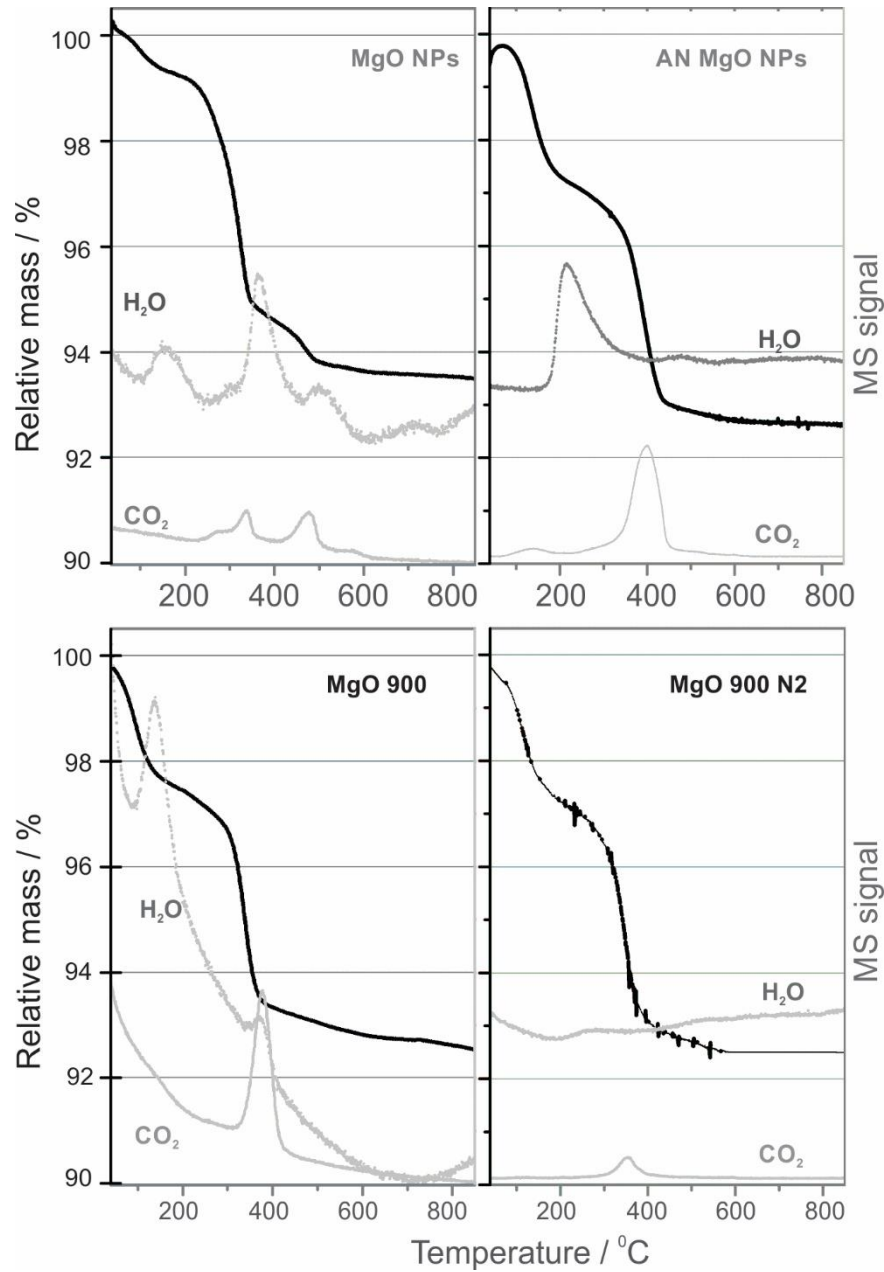


Figure 4.8: TGA determined mass loss profiles of the studied MgO powders coupled with the MS chemical analysis of released gasses.

The observed surface defect peculiarities of the studied MgO powders governed their specific surface area differences. MgO 900 had the lowest BET value ($6.6 \text{ m}^2/\text{g}$) due to absence of either kinks/di-vacancies or oxygen vacancies. MgO 900 N2, with the same morphology as MgO 900 microrods, exhibited a high concentration of oxygen vacancies and oxygen vacancy dimmers, which resulted in an increase of BET to $14 \text{ m}^2/\text{g}$. MgO NPs had the highest BET value due to a high abundance of kinks and step di-vacancies ($35 \text{ m}^2/\text{g}$). Its thermal annealing in air caused the surface reorganization. As a result, AN MgO NPs had a higher concentration of 4C oxygen atoms and reduced amount of kinks and step di-vacancies, which caused the reduction of BET area to $32 \text{ m}^2/\text{g}$.

4.1.2 Antibacterial properties of nano-textured MgO microrods

The correlations of antibacterial properties of MgO to (i) the morphology of the MgO microrods as well as to their surface defects peculiarities and (ii) the ROS generation were investigated.

The nano-textured MgO 900 microrods exhibited a strong bactericidal potential when finely dispersed in the MH broth medium. The evaluated MBC was equal to 0.5 mg/ml (Fig. 4.9a) and was significantly different to the MBC of other nano-textured microrods (MgO 700, MgO 800 and MgO 1000), which were 0.6 mg/ml (Fig. 4.9a). The MgO microrods which appeared smooth (MgO 400 and MgO 600) had much higher MBCs of 1.0 and 0.8 mg/ml, i.e. exhibited far weaker bactericidal potential against *E. coli*. Thus, the nano-texturing of the MgO microrods successfully improved their antibacterial activity. The optimal antibacterial activity was achieved for MgO 900, and these particles were more efficient than the commercial MgO NPs (MBC = 0.6 mg/ml).

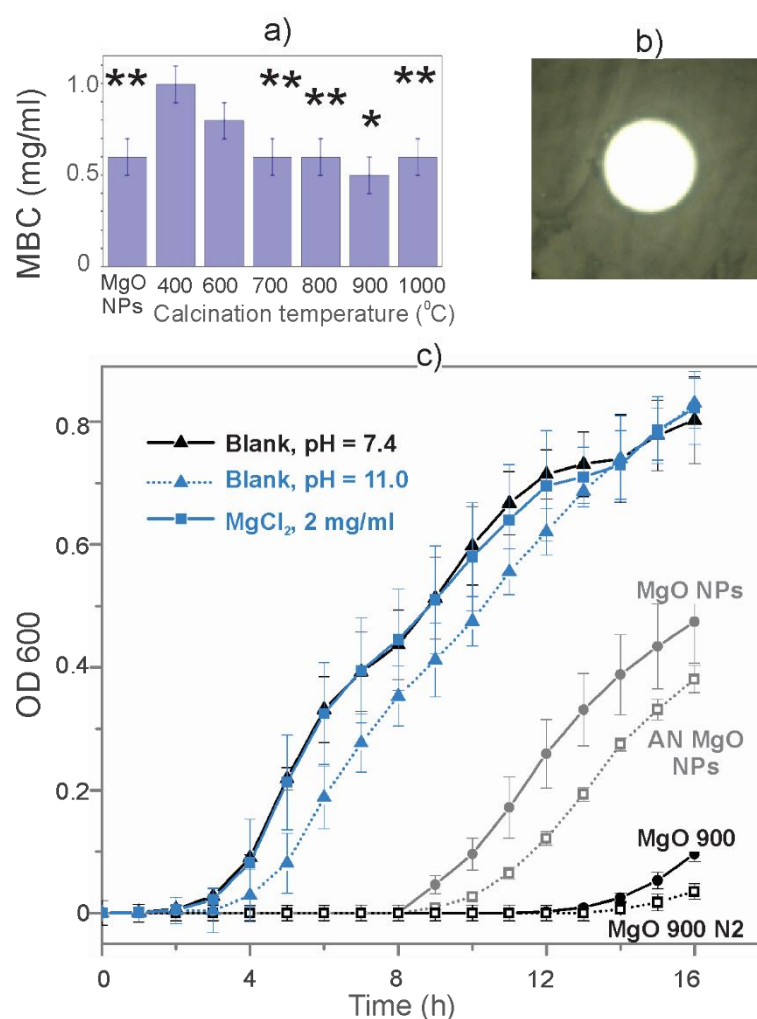


Figure 4.9: a) MBC for the various MgO powders (* and **significantly differ, $p < 0.05$); b) Confluent growth of *E. coli* around nano-textured MgO microrods after disc-diffusion test; c) The growth kinetics of *E. coli* in the MH medium.

When exposed to an aqueous environment, MgO is immediately hydrolysed and partially dissolved which causes a pH increase. The growth kinetics of the *E. coli* in the presence of Mg^{2+} ions (Fig. 4.9c, blue line with squares) and in basic pH solution (Fig. 4.9c

blue dotted line with triangles) was matched to the growth kinetics of the control (Fig. 4.9c bacteria in MH broth, black line with triangles), i.e. neither Mg^{2+} ions nor pH rise did not influence the *E. coli* growth kinetics. Together with an absence of inhibition zone in the disc-diffusion experiment (Fig. 4.9b), these results confirmed the contact-based mechanism of the antibacterial action of the nano-textured MgO microrods.

The study of the bacteria growth kinetics showed that the MgO suspensions with a concentration of particles equal to 0.4 mg/ml affected the *E. coli* growth, i.e. the bacteria proliferation was slowed (Fig. 4.9c). The nano-textured MgO 900 microrods (solid black lines) were more efficient than the commercial MgO NPs (dotted grey lines). AN MgO NPs were also slightly more efficient in inhibition of *E. coli* growth kinetics than the MgO NPs while MgO 900 N2 proved to be more efficacious than MgO 900. Thus, MgO surface defects peculiarities influenced the antibacterial activity.

The same trends in the antibacterial activity were obtained when the lethal potential of the studied MgO powders was determined by monitoring the ratio of live bacteria as a function of the exposure time by the means of *BacLight* Live/dead fluorescent assay (Fig. 4.10a). I observed that MgO 900 N2 exhibited the strongest lethal potential, as just 1% of *E. coli* were viable after 24 h (Fig. 4.10a, black dotted line) in comparison with 4%, 6% and 13 % when bacteria were exposed to MgO 900, AN MgO NPs and MgO NPs, respectively. The kinetics of the elimination of viable bacteria is very important, especially in antimicrobials with contact-based mechanism. It is desirable to eliminate bacteria as fast as possible. For this reason, I calculated the initial lethal rate (r_0) of MgO powders. The procedure included fitting with decaying rational function and calculation of the first derivative at point $t = 0$. The obtained values (Fig. 4.10b) showed that at the initial stage of exposure MgO 900 N2 ($r_0 = 0.413 \text{ h}^{-1}$) particles eliminate bacteria more than two times faster than MgO NPs ($r_0 = 0.190 \text{ h}^{-1}$). The thermal annealing resulted in a slight increase in the initial lethal rate, as AN MgO NPs eliminated *E. coli* with $r_0 = 0.223 \text{ h}^{-1}$. The impact of thermal processing atmosphere was significant, as MgO 900 prepared in air exhibited 21 % reduced initial lethal rate (0.333 vs 0.413 h^{-1}) compared to MgO 900 N2 prepared in nitrogen atmosphere. However, they were still significantly more powerful than both, MgO NPs and AN MgO NPs.

The labelling of bacteria by the *BacLight* fluorescent dyes enabled their observation under the microscope, which provided a micro-scale view of the bacterial viability upon the exposure to MgO. The SYTO 9, a green-fluorescent dye, is permeable through bacterial wall and binds to nucleic acids of both dead and viable cells, while the PI, a red-fluorescence dye, only penetrates the mechanically destroyed or damaged bacterial walls and binds to the nucleic acids of dead bacteria [139]. After 24 h in MH broth the bacteria (Fig. 4.10c, control) were observed as green dots, which was evidence that the majority of the bacteria were alive. After 24 h of exposure to nano-textured MgO in the MH broth, green and red dots overlapped, which proved that the vast majority of the bacteria were dead (Fig. 4.10c). However, the green and red dots were observed smaller than in the control experiment. SEM image of the *E. coli* after 24 h in the MH broth revealed their cylindrical morphology (Fig. 4.10d, control). The *E. coli* exposed to MgO had a destroyed wall (Fig. 4.10d) and the intracellular content leaked out. As a result, the dyes from the kit were bonded to the specific sites in a less-localized manner, when compared to the control, i.e., the signal was less localized (Fig. 4.10d). These experiments indicated strong interaction of the MgO with a bacterial wall.

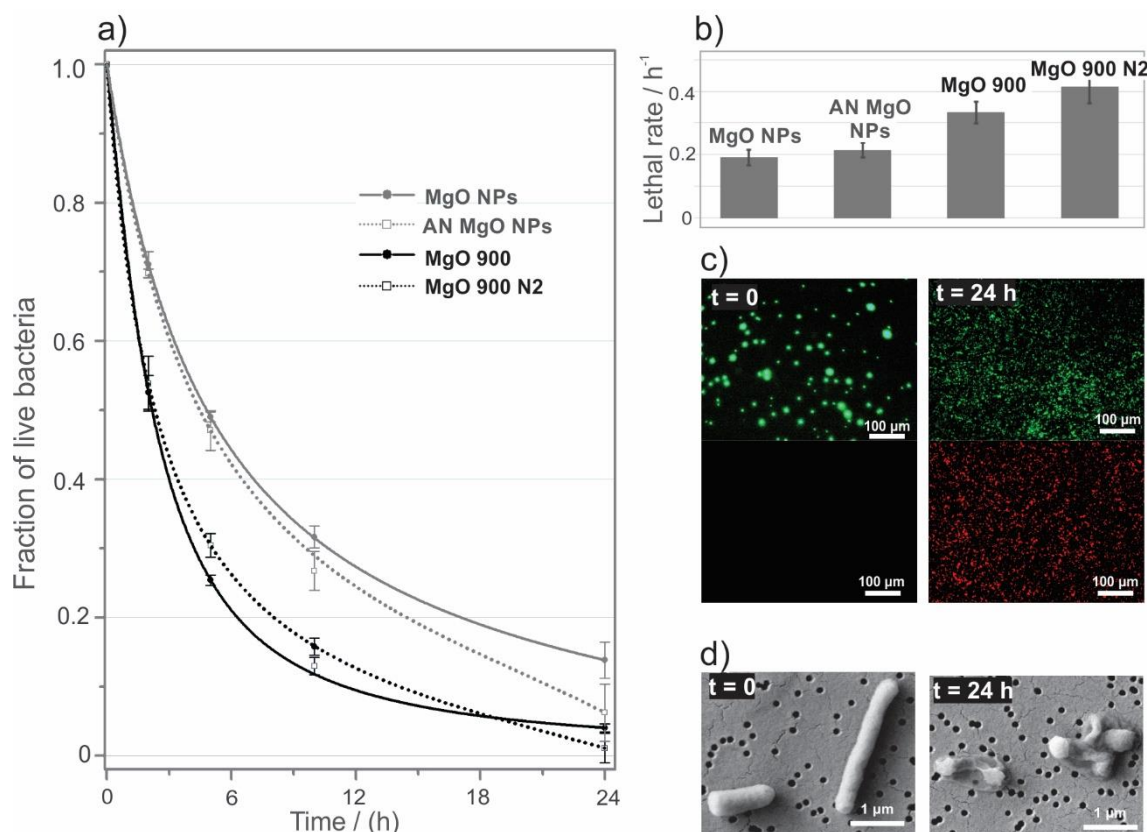


Figure 4.10: a) The fraction of viable *E. coli* versus the exposure time to the MgO particles; b) the initial lethal rate against *E. coli* of the studied MgO powders; c) fluorescent imaging and d) SEM imaging of *E. coli* after 24 h of exposure to MgO 900.

To complement the measurements of antibacterial activity, we measured the ROS generation of the MgO powders *in situ* upon incubation in physiological solution without (Fig. 4.11a, b) and with the bacteria (Fig. 4.11c, d) by the means of the fluorescent DHR assay. Among the tested MgO powders, just the MgO NPs exhibited a significant generation of the ROS species (Fig. 4.11a, b). However, when the bacteria were added, a ROS generation was observed for all of the tested MgO powders (Fig. 4.11c, d). The positive control, consisting of H_2O_2 and *E. coli* did not provide any signal, which indicated the ability of the *E. coli* to metabolise the H_2O_2 into products not able to oxidize DHR.

The EPR spectroscopy was performed as a complementary method to the DHR fluorescence assay. With incubation at 37°C , the DMPO-OH signal within the UV-treated 5-mM H_2O_2 solution was losing intensity (Fig. 4.12). This was in accordance with the previously reported compromised stability of the DMPO-OH radical [140]. There was no ROS generation detected in the suspension of MgO NPs and the DMPO before incubation (Fig. 4.12a). After 15 min and 30 min of incubation, DMPO-OH radicals were detected in the suspension of MgO NPs (Fig. 4.12b, c) and, unlike the H_2O_2 , the signal was gaining intensity. In contrast, there was no EPR signal detected in the suspension of MgO 900 particles, neither before nor after the incubation (Fig. 4.12). The introduction of bacteria to the MgO 900 suspension resulted in the emergence of the EPR signal after 30 minutes of incubation at 37°C (Fig. 4.12c), while there was no EPR signal, neither before nor after 15 minutes of incubation (Fig. 4.12a, b).

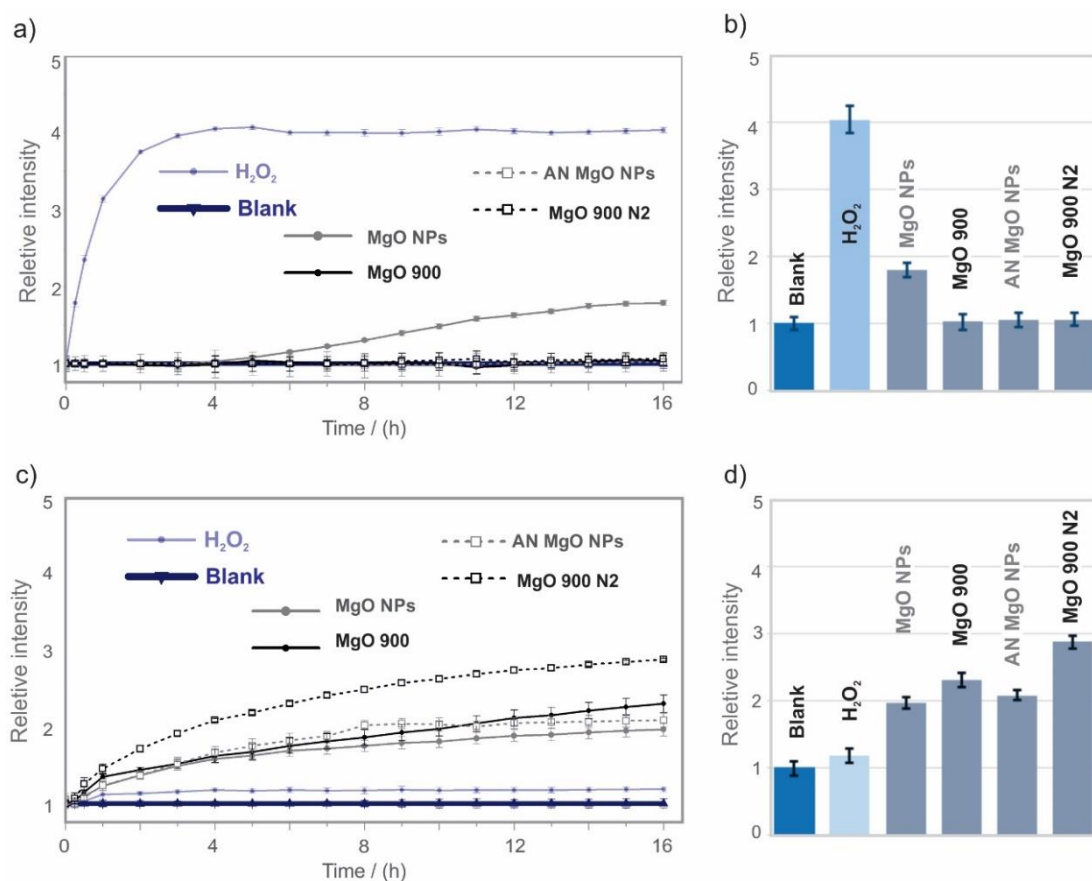


Figure 4.11: The *in-situ* generation of ROS species of the studied MgO powders during incubation in physiological solution either a) without or c) with bacteria; The total amount of generated ROS species after 16 h incubation of the studied MgO powders in physiological solution either b) without or d) with bacteria. The 5 mM solution of H_2O_2 was used as positive control. The results were provided relative to the blank solution.

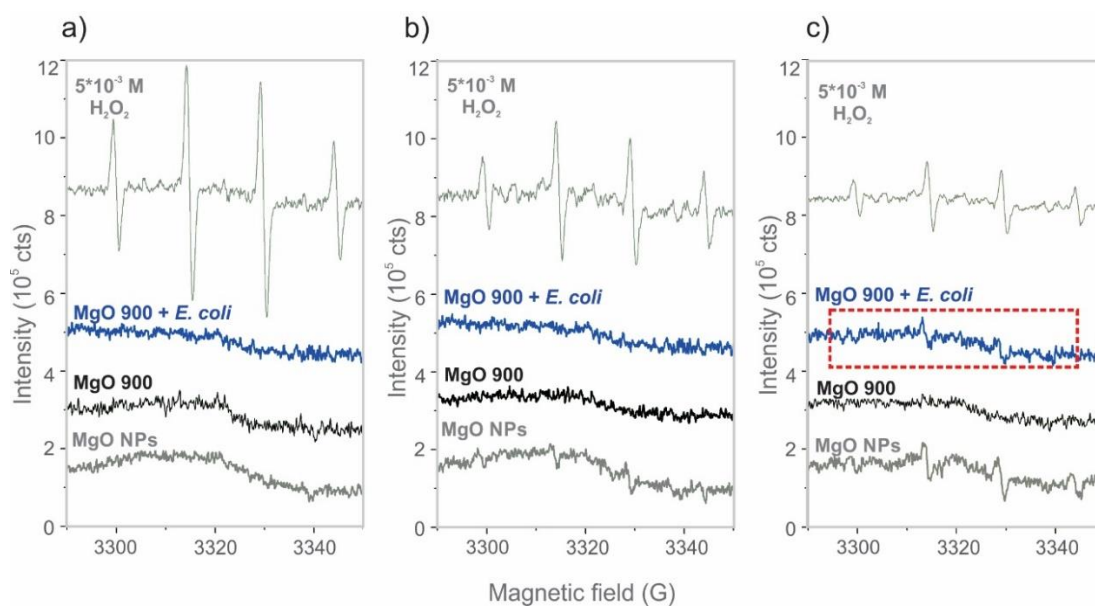


Figure 4.12: The EPR spectra a) at the beginning b) after 15 minutes and c) after 30 minutes of incubation.

Results obtained by the means of *in-situ* EPR method were in accordance with the results obtained by means of the fluorescent DHR method and together provided proofs that ROS production is not an inherent property of MgO, but rather that these species are generated during :

1. MgO/bacteria interactions as a side-product, i.e., rather a consequence of than the reason for the *E. coli* death;
2. MgO particle hydrolysis as a side product, but only when the MgO surface contained low-coordinated oxygen sites (kinks, step di-vacancies).

The confirmation of observed role of ROS as a side product of the MgO/bacteria interactions included an evaluation of the *E. coli* susceptibility to MgO in the presence of ascorbic acid, as the ascorbate ion was recognized for its ROS inhibition [141]. MgO (basic oxide) tends to react with ascorbic acid, which could result in a reduced concentration of MgO solid form. In our case, the dissolution was not complete and the tested suspension containing MgO, Ascorbic acid and bacteria was still slightly basic. To evaluate impact of excessive dissolution of MgO in the presence of ascorbic acid, an MgO-NPs/HCl mixture with an equimolar acid concentration was used as a positive control. The results indicated that MgO NPs completely inhibited the growth of *E. coli* at a concentration of 0.6 mg/ml (Fig. 4.13). Although the growth of *E. coli* was still observed when exposed to the MgO-NPs/ascorbic acid mixture at MgO concentrations of 0.6 mg/ml and 0.8 mg/ml, similar profiles of *E. coli* growth were observed when exposed to the MgO-NPs/HCl mixture (Fig. 4.13). These observations confirmed that the partly reduced antibacterial activity of the MgO/ascorbic acid mixture was a consequence of the reduced MgO solid form concentration due to its reaction with ascorbic acid. These results provided another proof that the ROS species were a side product of the MgO/bacteria interactions.

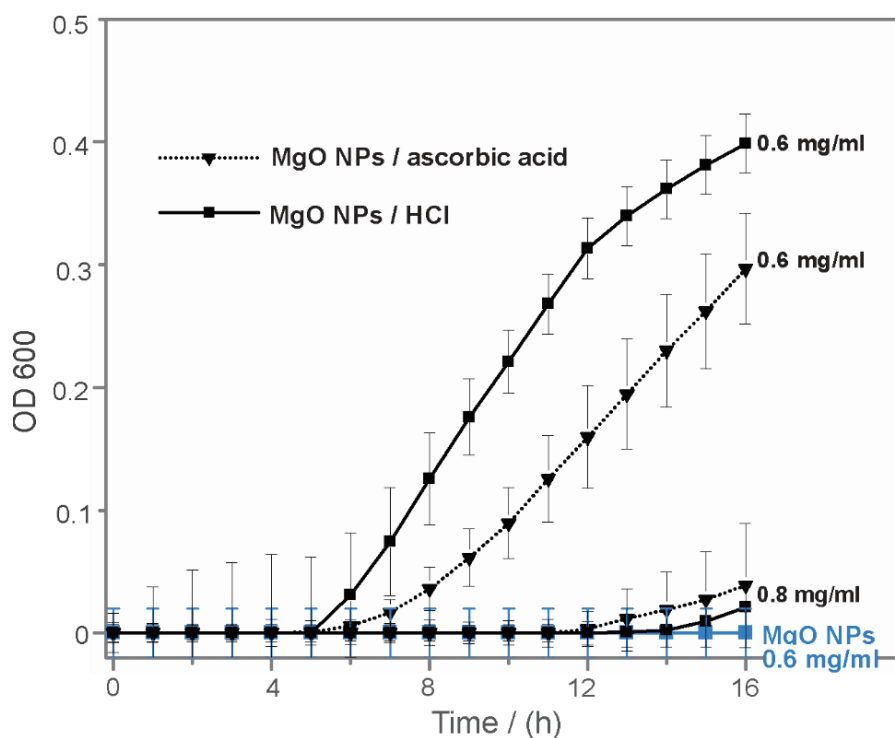


Figure 4. 13: The growth kinetics of *E. coli* in the MH medium in the presence of the MgO/ascorbic acid mixture. The concentration of ascorbic acid was equal to 1 mg/ml. Equimolar MgO/HCl mixture was used as a control.

4.1.3 The MgO hydrolysis and oxide to hydroxide transformation

The kinetics of MgO hydrolysis and oxide to hydroxide transformation was evaluated in four different media, which are commonly used for the testing of either study MgO antibacterial activity or to perform toxicological studies on human cell lines.

The XRD diffractograms of MgO powders were collected after exposure to physiological solution for various time intervals. For all of the studied powders, the transformation from the oxide-phase to the hydroxide-phase (JCS Card 44-1482) was confirmed (Fig. 4.14) within 16 h of exposure.

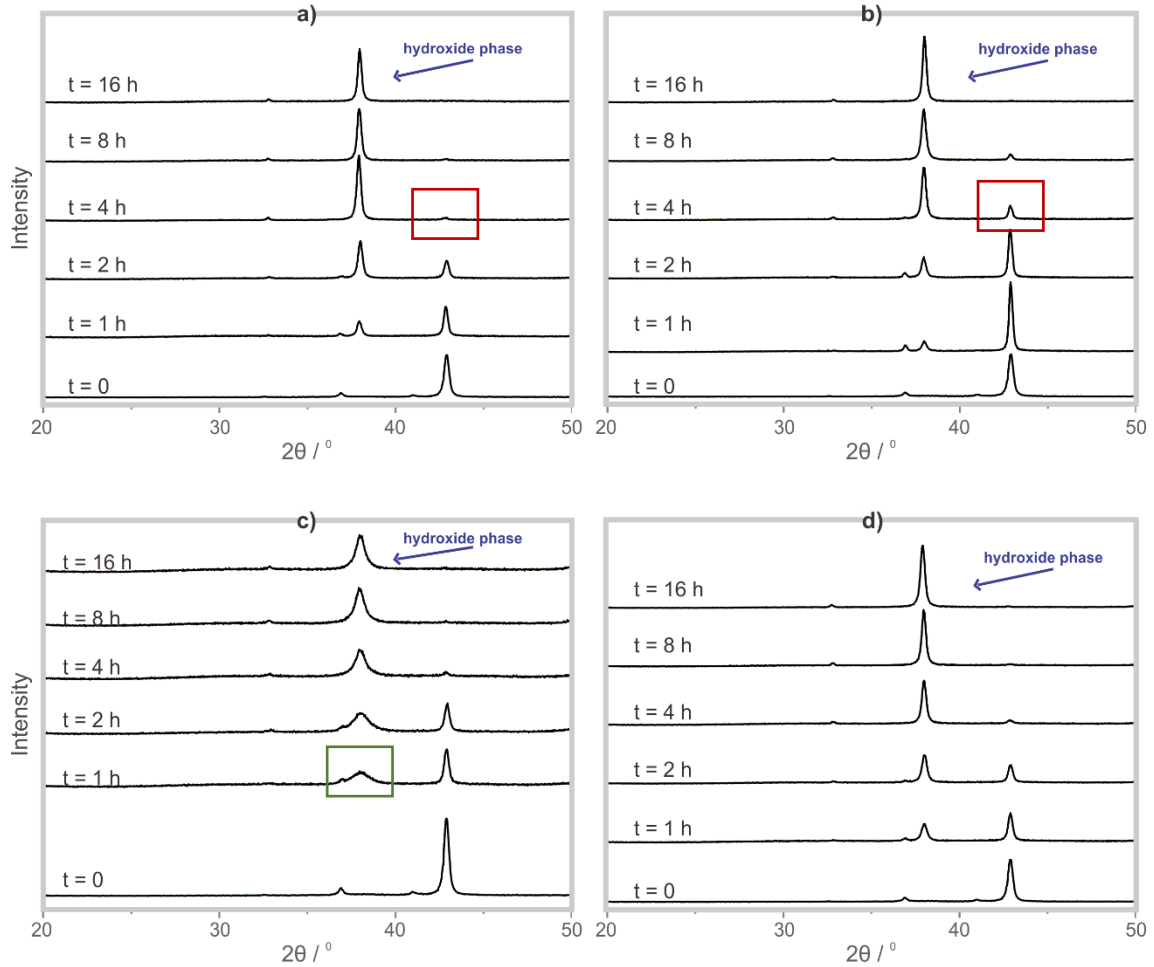


Figure 4.14: The XRD diffractograms of the a) MgO 900 microrods, b) MgO 900 N2 microrods, c) MgO NPs and d) AN MgO NPs as a function of exposure time to the physiological solution.

However, through the process of the phase transformation some differences were identified. Firstly, a significant amount of MgO phase was observed after a 4 h long exposure of MgO 900 N2 (Fig. 4.14 b, indicated by red square) to the physiological solution. On the contrary, only traces of MgO phase observed in the case of MgO 900 (Fig. 4.14a, indicated by red square). This indicated differences in the kinetics of phase transformation among the studied MgO powders. Secondly, the XRD peak representing (101) crystals planes of $\text{Mg}(\text{OH})_2$ appeared wider in the case of MgO NPs than in the case of other three powders (Fig. 4.14c, indicated by green square). This indicated the difference in the crystal growth of $\text{Mg}(\text{OH})_2$ particles during the oxide-hydroxide transformation among the studied MgO powders.

The comprehensive study of the observed phenomena included quantification of the oxide-to-hydroxide phase transformation (Fig. 4.15a). The calculation of the phase conversion (x) using the “HighScore Plus” software was performed. The most pronounced difference in the phase conversion among the studied MgO powders was measured in the initial stage (within 1 h) of exposure. It was decreasing from 45 % (MgO NPs), 34 % (AN MgO NPs), 29 % (MgO 900) to just 9 % (MgO 900 N2). The observed trend was completely opposite to the trend in antibacterial activity of the MgO powders (Fig. 4.10c), where the MgO 900 N2 particles were the most potent while the MgO NPs were the least potent against *E. coli*. Other identified difference was in the shape of phase transformation curve. After the initial stage (from 1 h to 4 h long exposure), the conversion curves were steeper in the case of the MgO 900 and the MgO 900 N2 particles than for the MgO NPs and the AN MgO NPs. We related this difference to the particle morphology because just in the case of both, MgO NPs and AN MgO NPs, large agglomerates (up to 5 μm) were observed by the means of SEM (Fig. 4.4).

The crystallite size calculation by the means of Scherrer equation indicated that the average crystallite of formed hydroxide phase was different between the studied MgO powders (Fig. 4.15b). The crystallites of hydroxide phase formed from MgO NPs were increasing in size as it was varying from 9 nm ($t = 1\text{ h}$) to 15 nm ($t = 16\text{ h}$). In contrast, the crystallite size of formed hydroxide phase was rather constant over exposure time for the other three studied powders (AN MgO NPs, MgO 900 and MgO 900 N2) and was equal to 30 nm (Fig. 4.15b).

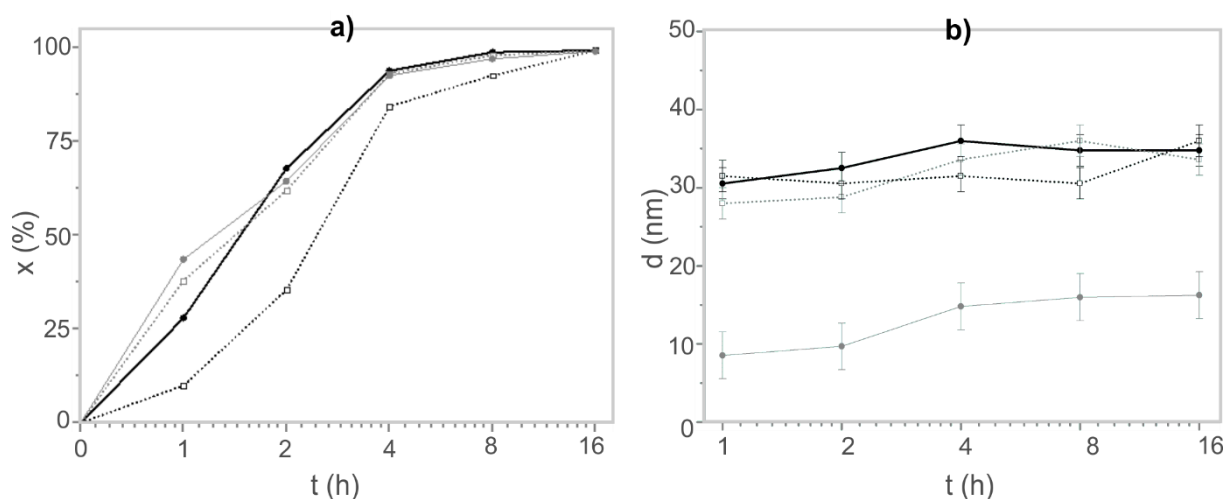


Figure 4.15: a) The conversion of oxide to hydroxide phase as a function of exposure time to the physiological solution for the studied MgO powders; b) The average crystallite size of the formed magnesium hydroxide phase calculated using the Scherrer equation as a function of exposure time to the physiological solution for the various MgO powders.

AN MgO NPs and MgO NPS exhibited a similar morphology upon the SEM observation (Fig. 4.4c, d), but the difference in the crystallite size of formed hydroxide was three fold in the initial stage of their exposure to physiological solution (Fig. 4.15b). In contrast, the exposure of AN MgO NPs and MgO 900 particles led to $\text{Mg}(\text{OH})_2$ crystallites with the same size even though their morphology was different (Fig. 4.4a, d). Thus, the observed differences in the crystallite size of formed hydroxide phase could not relate to differences in morphology of the starting MgO powders.

The morphological differences of formed $\text{Mg}(\text{OH})_2$ were confirmed by the means of SEM. After 2 hours of exposure, MgO 900 microrods were partially covered by the round

magnesium hydroxide discs (Fig. 4.16c). This matched well with the XRD study of phase transition kinetics (Fig. 4.14), which indicated a mixture of both oxide and hydroxide phases. After 24 h of the exposure, the process of phase transformation was completed and the magnesium hydroxide discs changed shape and became hexagonal. The discs were 300 nm in diameter and 10 nm in thickness and were assembled in microrods, similar as original MgO 900 microrod particles (Fig. 4.16a, e). Thus, the microrod morphology was preserved.

Similarly, the growth of the magnesium hydroxide particles also occurred at the surface of MgO NPs agglomerates, as observed after 2 h of exposure to physiological solution (Fig. 4.16d). At the end of the MgO NPs' exposure ($t = 24\text{h}$) to physiological solution, the formed magnesium hydroxide discs appeared smaller than the discs from MgO 900 in both, width and thickness (Fig. 4.16e, f). The formed $\text{Mg}(\text{OH})_2$ nanodiscs were assembled into the agglomerates (Fig. 4.16f) same as MgO NPs (Fig. 4.4c).

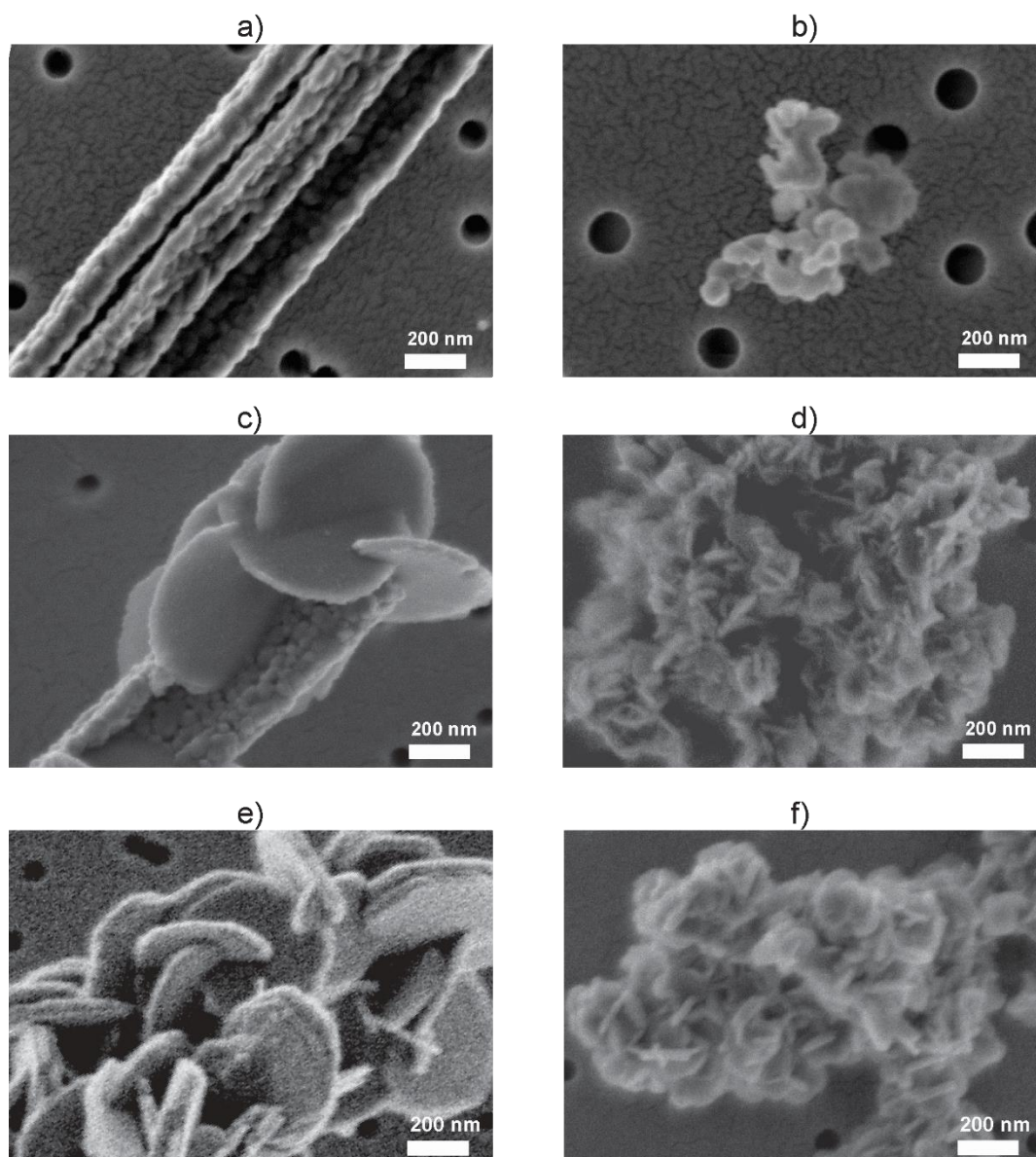


Figure 4.16: The SEM imaging of the MgO 900 a) before, c) after 2 h of exposure and e) after 24 h of exposure to the physiological solution; The SEM imaging of the MgO NPs b) before, d) after 2 h of exposure and f) after 24 h of exposure to the physiological solution

The XRD analysis of the MgO powders after the exposure to PBS was performed and profound differences in the phase transformation kinetics were observed when compared to the exposure in the physiological solution. Firstly, the transformation from the oxide-phase to the hydroxide-phase required a longer time to proceed, i.e. 24 h instead of 8 h for MgO 900 (Fig. 4.17 a) and 96 h instead of 8 h for MgO NPs (Fig. 4.17 b).

Secondly, the difference in the size of the formed $\text{Mg}(\text{OH})_2$ crystallites in PBS was not statistically significant for both of the tested powders (9.4 ± 2.0 nm from MgO 900 and 9.9 ± 2.0 nm from MgO NPs, Fig. 4.17). This was opposite to the crystal growth in the physiological solution where the average crystallite sizes of $\text{Mg}(\text{OH})_2$ discs were equal to 30 nm (obtained from MgO 900) and 15 nm (obtained from MgO NPs, Fig. 4.15).

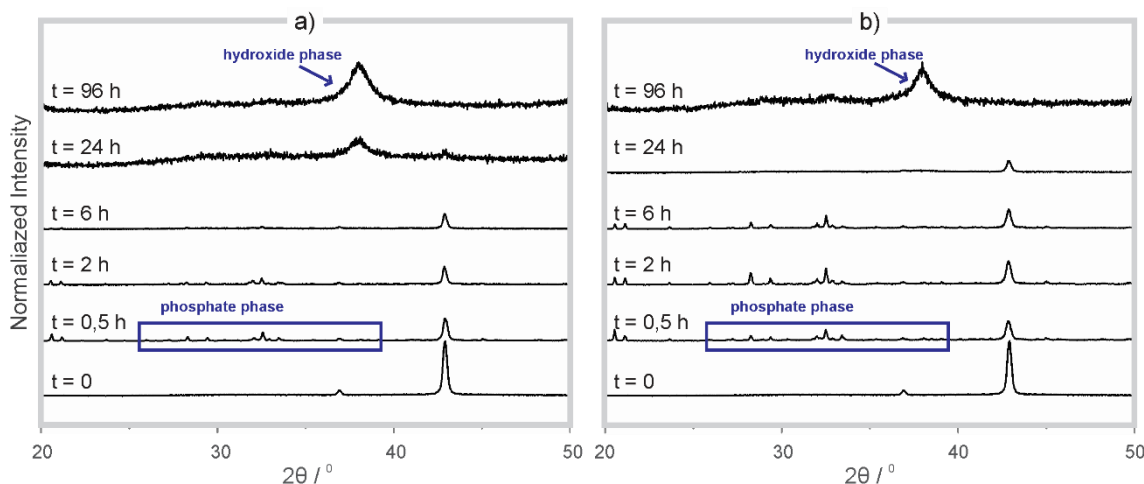


Figure 4.17: The XRD diffractograms of the a) MgO 900 microrods and b) MgO NPs as a function of exposure time to the PBS media.

Most importantly, the phase transformation of both studied MgO powders included the generation of phosphate phase (Fig. 4.17). The exact phase composition of the phosphate phase could not be matched to any within ICSD base. The closest matches were orthorhombic magnesium hydrogen phosphate hydrate ($\text{MgHPO}_4 \cdot 3\text{H}_2\text{O}$, ICSD 70-2345) and potassium magnesium phosphate (KMgPO_4 , ICSD 50-0151). The XRD maxima corresponding to the phosphate phase were the most pronounced after 30 min of exposure and were diminishing in the intensity. After 24 h of the exposure of the MgO powders to the PBS, no traces of a crystal phosphate phase were found at the XRD spectra (Fig. 4.17).

The identified phenomena from the XRD spectra were further studied by the means of SEM and TEM. When both, MgO 900 and MgO NPs, were exposed to the PBS for 2 h, the smooth 1 D microrods were observed (Fig. 4.18a). The SAED analysis proved their monocrystalline nature and orthorhombic crystal structure (Fig. 4.19a, upper-left corner).

The EDS spectra of formed phosphate phase indicated the presence of K, Na, Mg, P and O (Fig. 4.19c, lower-right-hand corner). After 24 h of exposure of the MgO 900 to the PBS, the phase transformation was completed. The formed $\text{Mg}(\text{OH})_2$ nanocrystallites were in the shape of thin flakes and were assembled in microrods (Fig. 4.18c). Thus, particle morphology was preserved. The SAED confirmed that these particles consisted of magnesium hydroxide crystallites without any remainings of magnesium oxide phase (Fig. 4.19b, upper-left-hand corner). Except from $\text{Mg}(\text{OH})_2$ particles, agglomerates of thin platelets were identified upon TEM exposure. Their size was around 200 nm. The EDS spectra (Fig. 4.19b, lower-left-hand corner) indicated the presence of Mg, O and P while the SAED demonstrated an amorphous nature of these structures (Fig. 4.19b, lower-right-hand corner). I identified this phase as amorphous magnesium phosphate phase.

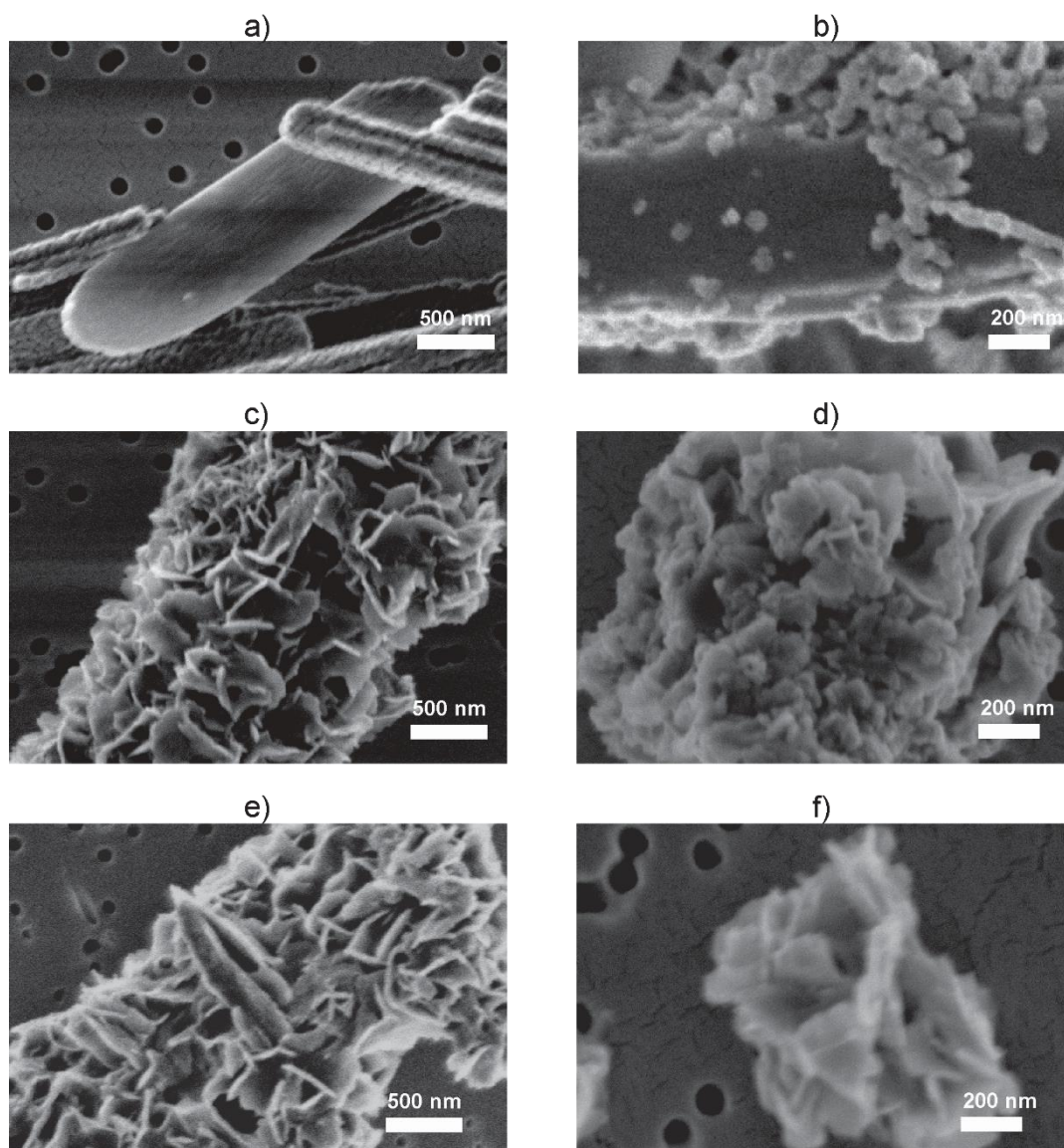


Figure 4.18: The SEM imaging of the MgO 900 after a) 2 h of exposure, c) 24 h of exposure and e) 96 h of exposure to the PBS medium; The SEM imaging of the MgO NPs after b) 2 h of exposure, d) 24 h of exposure and f) 96 h of exposure to the PBS medium.

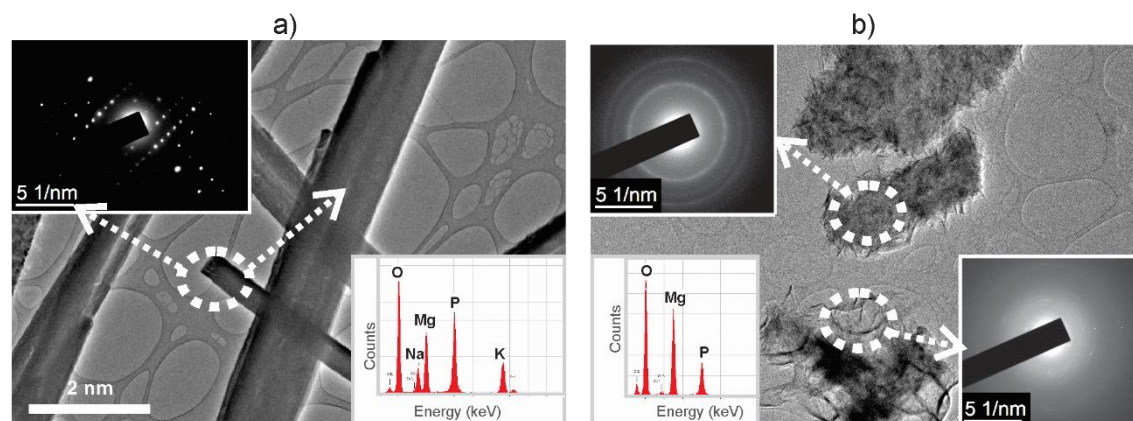


Figure 4.19: TEM, SAED and EDS study of the phosphate phase obtained after a) 2 h of exposure and b) 24 h of exposure of MgO 900 to the PBS media.

When MgO NPs were exposed to PBS solution for 24 h, agglomerates were observed (Fig. 4.18d) like during the phase transformation in physiological solution (Fig. 4.16f). They were covered with approximately 300 nm large thin platelets, which were identified as amorphous phosphate flakes. After 96 h, the large agglomerates of MgO NPs were transformed to the agglomerates of $\text{Mg}(\text{OH})_2$ thin flakes (Fig. 4.18f).

The measurement of MgO antibacterial activity was performed in the MH medium. Except from phosphate, sodium and chloride ions, it contains also amino acids and carbohydrates. Because of that, the process of MgO hydration and dissolution was also performed in MH broth medium.

Within 2 h of exposure to MH medium, the emergence of crystalline phase was observed for the both, MgO 900 and MgO NPs (Fig. 4.20). Similar to exposure in the PBS, this phase was not detected in the XRD within 24 h of the exposure, i.e. it was amorphized. However, the emergence of the hydroxide phase within 24 h of exposure was not observed for neither MgO 900 nor MgO NPs. Within 96 h of exposure, both MgO 900 and MgO became completely amorphized (Fig. 4.20).

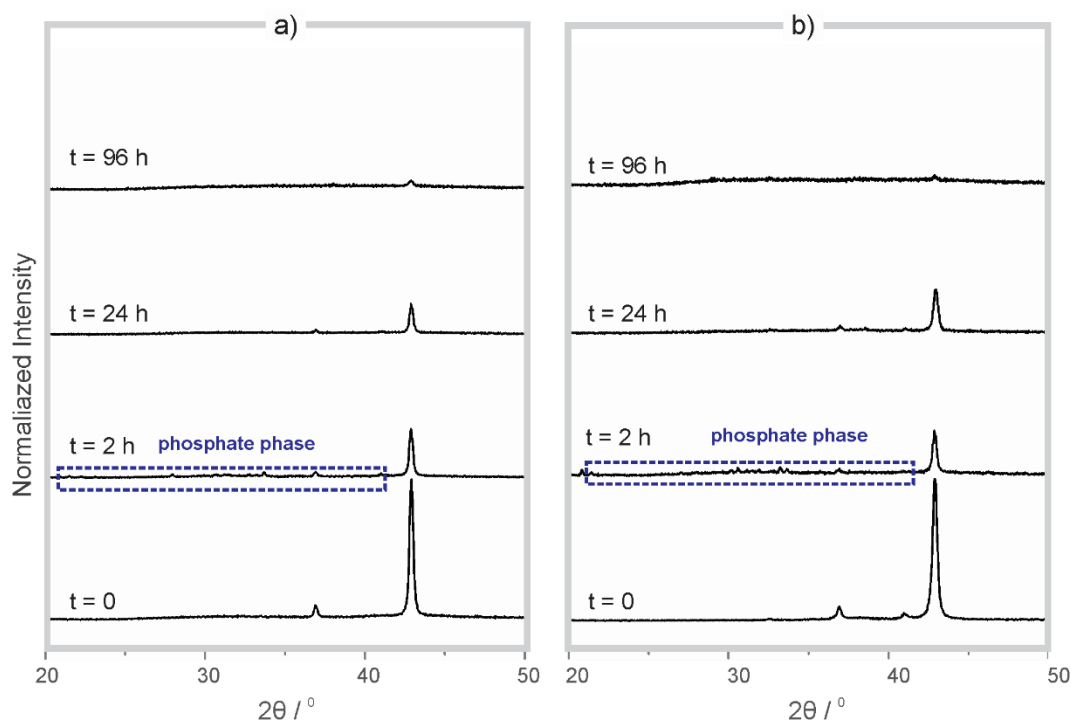


Figure 4.20: The XRD diffractograms of the a) MgO 900 microrods and b) MgO NPs as a function of exposure time to the MH broth media.

The further insight into the morphological changes during the MgO hydration in the MH broth medium was gained by the means of SEM. Within 2 h of exposure, MgO particles retained their morphology (Fig. 4.21a,b). However, the surfaces of both, nano-textured MgO 900 microrods and MgO NPs, were covered with a layer of adsorbates (Fig. 4.21a,b).

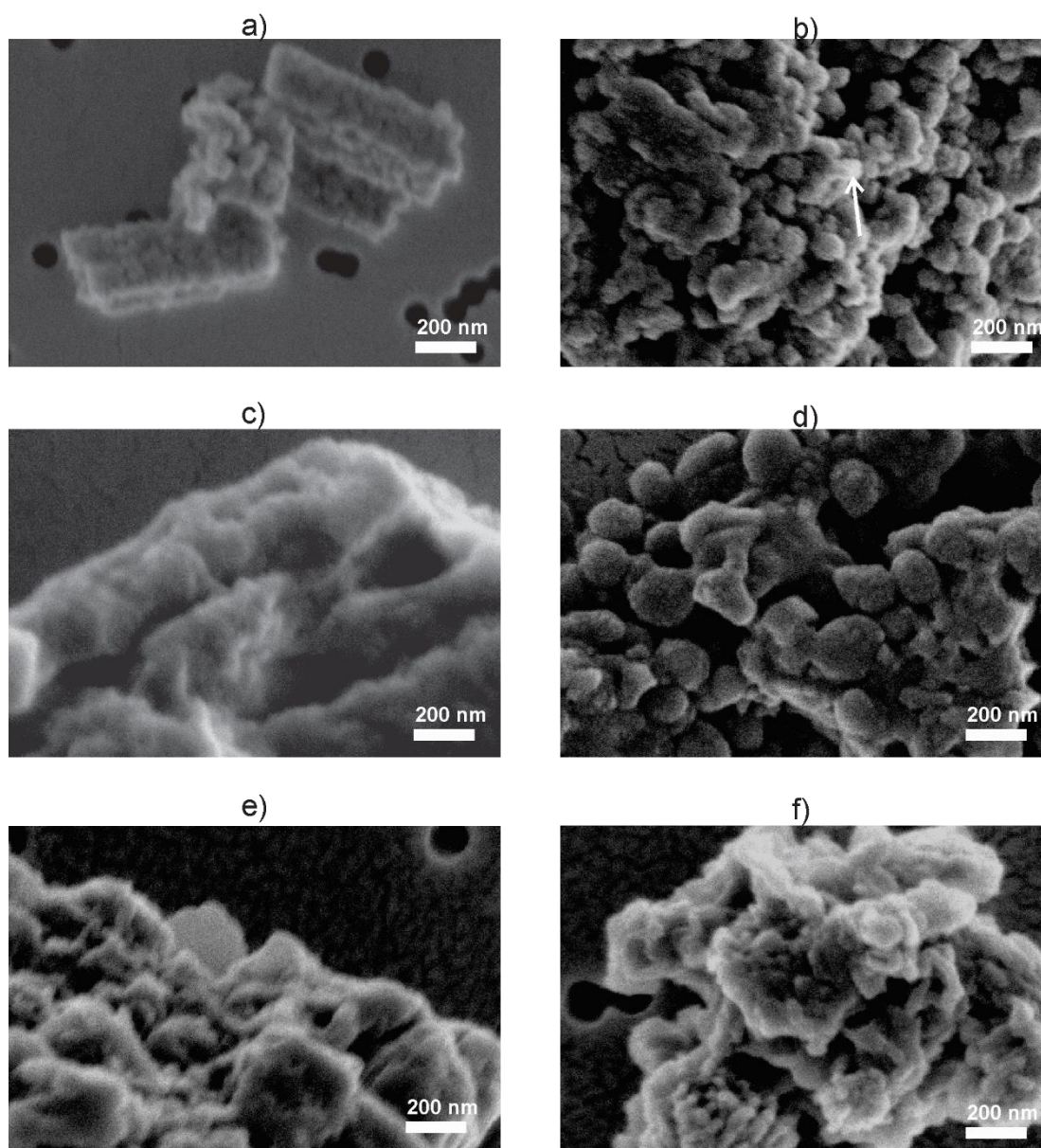


Figure 4.21: The SEM imaging of the MgO 900 after a) 2 h of exposure, c) 24 h of exposure and e) 96 h of exposure to the MH growth medium; The SEM imaging of the MgO NPs after b) 2 h of exposure, d) 24 h of exposure and f) 96 h of exposure to the MH growth medium.

These adsorbates originated from the MH medium. Within 24 h, the nano-textured MgO microrods were completely covered with the adsorbates, so that their nano-texturing was no longer observable by means of SEM (Fig. 4.21c). The MgO NPs appeared different after 24 h of exposure. Instead of agglomerates of NPs with a size of 30 nm, they consisted out of the agglomerates containing the NPs of 100 nm (Fig. 4.21d). I explained this phenomena by the encapsulation of several MgO NPs with the adsorbates from the MH broth medium. These adsorbates were still present at the surface of both powders after 96 h (Fig. 4.21f).

The application of MgO as an antibacterial agent within human body would lead to interactions with the surrounding body-fluids. The MgO would be surrounded by the

variety of ions, proteins, vitamins, growth factors, etc. Because of that, the behaviour of the MgO powders in the cell-culture growth medium (CellM) was evaluated.

In contrast to the MH broth medium, I did not identify the formation of any crystalline phosphate phase when the MgO powders were exposed to CellM for 2 h (Fig. 4.22). Within 24 h, there were no changes in the phase composition of neither MgO 900 microrods nor MgO NPs (Fig. 4.22). However, after 96 h the phase transformation from oxide to the hydroxide phase was observed. It was more pronounced for MgO 900 ($x = 0.3$) than for the MgO NPs ($x = 0.06$) (Fig. 4.22).

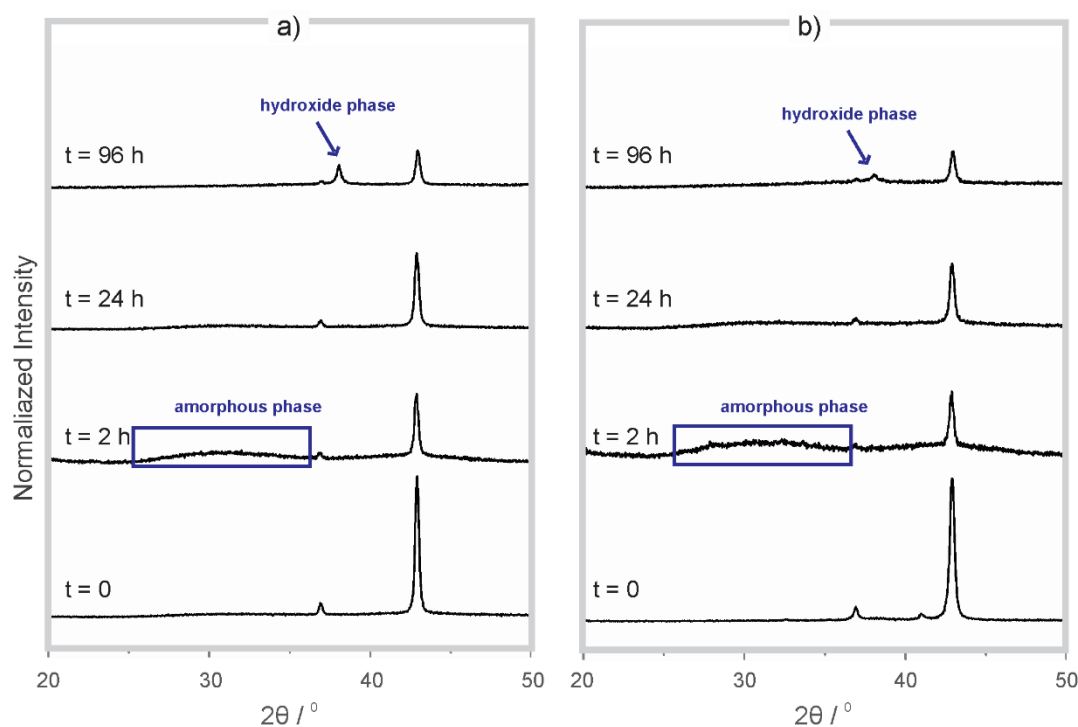


Figure 4.22: The XRD diffractograms of the a) MgO 900 microrods and b) MgO NPs as a function of exposure time to the cell-culture growth media.

The morphological changes upon the exposure to the CellM were evaluated by the means of SEM. Within either 2 h or 24 h of exposure, the nano-textured MgO 900 microrods were covered by an amorphous layer of the adsorbates from CellM (Fig. 4.23a). Similarly, MgO NPs were in the form of large agglomerates after either 2 h or 24 h long exposures in the CellM (Fig. 4.23b). The adsorbates were identified on the SEM images (Fig. 4.23a, c). However, both MgO 900 microrods and MgO NPs were strongly agglomerated within 96 h of exposure and required to be crushed in the mortar in order to prepare specimens for SEM morphology (Fig. 4.23e, f). The distinct microrod morphology was lost after nano-textured MgO particles were exposed for 96 h to the CellM (Fig. 4.23e).

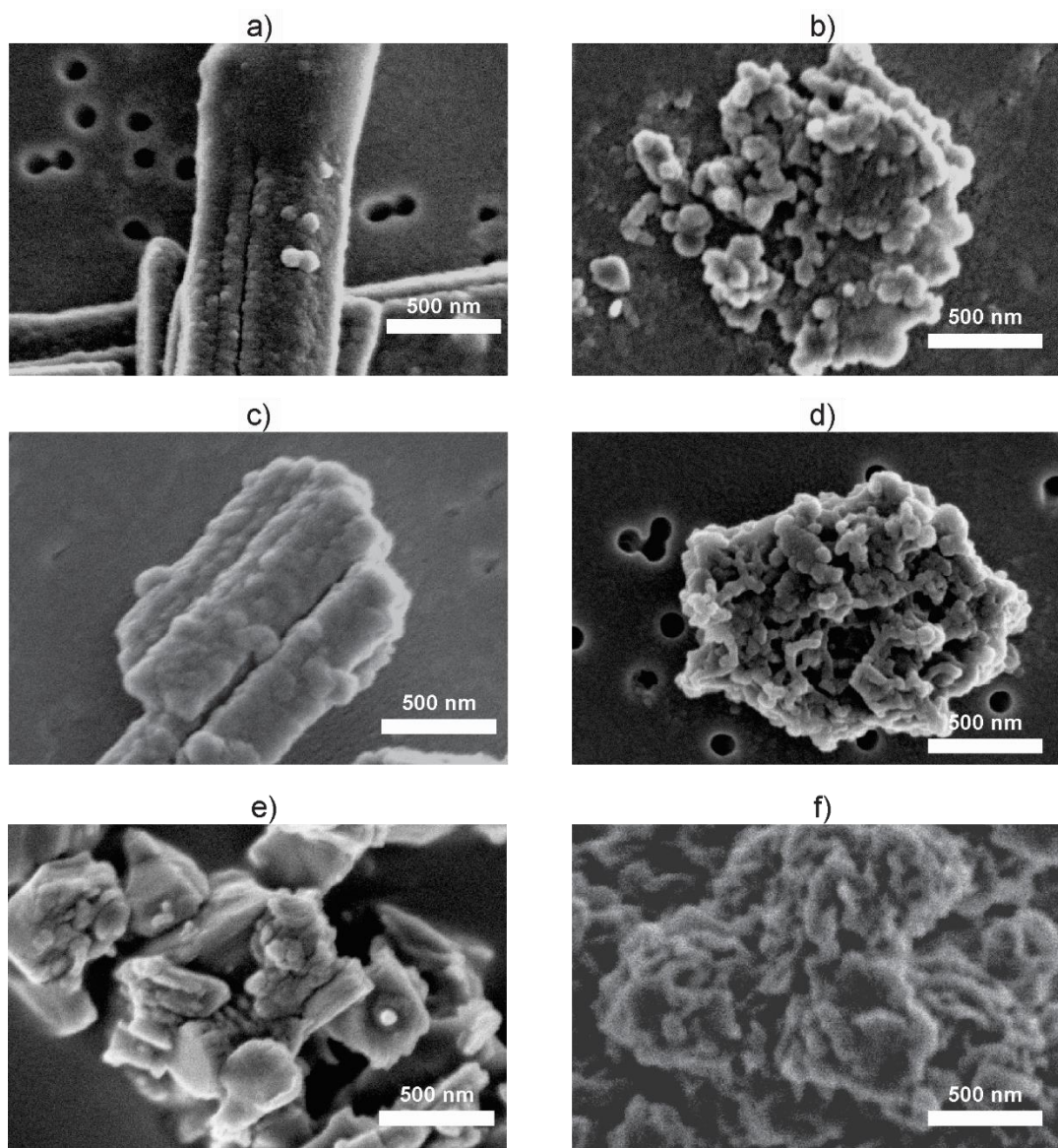


Figure 4.23: The SEM imaging of the MgO 900 after a) 2 h of exposure, c) 24 h of exposure and e) 96 h of exposure to the cell-culture growth medium; The SEM imaging of the MgO NPs after b) 2 h of exposure, d) 24 h of exposure and f) 96 h of exposure to the cell-culture growth medium.

During exposure to all four evaluated aqueous media, the hydrolysis of MgO was identified. It was a dynamical process and the growth of hydroxide phase proceeded at the surface of MgO. The presence of phosphate ions and/or organic molecules (proteins, carbohydrates, amino acids, etc.) has a strong influence on the hydrolysis kinetics as well as the growth of the hydroxide phase.

4.2 The Dissolution and Antibacterial Properties of the Nanostructured Vanadium Pentoxide

Either pure or intercalated nanostructured V_2O_5 oxides were prepared by the means of hydrothermal synthesis. During the process, the correlation between V_2O_5 physicochemical properties, dissolution kinetics and antibacterial properties was studied. The commercial V_2O_5 MPs were used as reference materials.

4.2.1 Physicochemical properties of the nanostructured V_2O_5

The impact of various dopants (H_2O , K) within the structure of nanostructured V_2O_5 at the phase composition and particle morphology was evaluated.

The XRDs spectra confirmed the phase homogeneity of the prepared V_2O_5 powders (Fig. 4.24). The XRD patterns of both, V_2O_5 MPs and V_2O_5 NWs, were matched to the crystal structure of the orthorhombic of vanadium pentoxide (JCPDS card 85-0601), i.e., they exhibit the same phase composition (Fig. 4.24). However, the XRD pattern of V_2O_5 NWs (Fig. 4.24) exhibited only (0kl) peaks, i.e., the diffraction peaks representing the (110), (101), (111), (130), (121), (141) and (102) crystallographic directions were not observed in the XRD pattern (Fig. 4.24). These findings indicate that the crystals of the V_2O_5 NWs display a preferential orientation, while the commercial V_2O_5 MPs are not preferentially oriented.

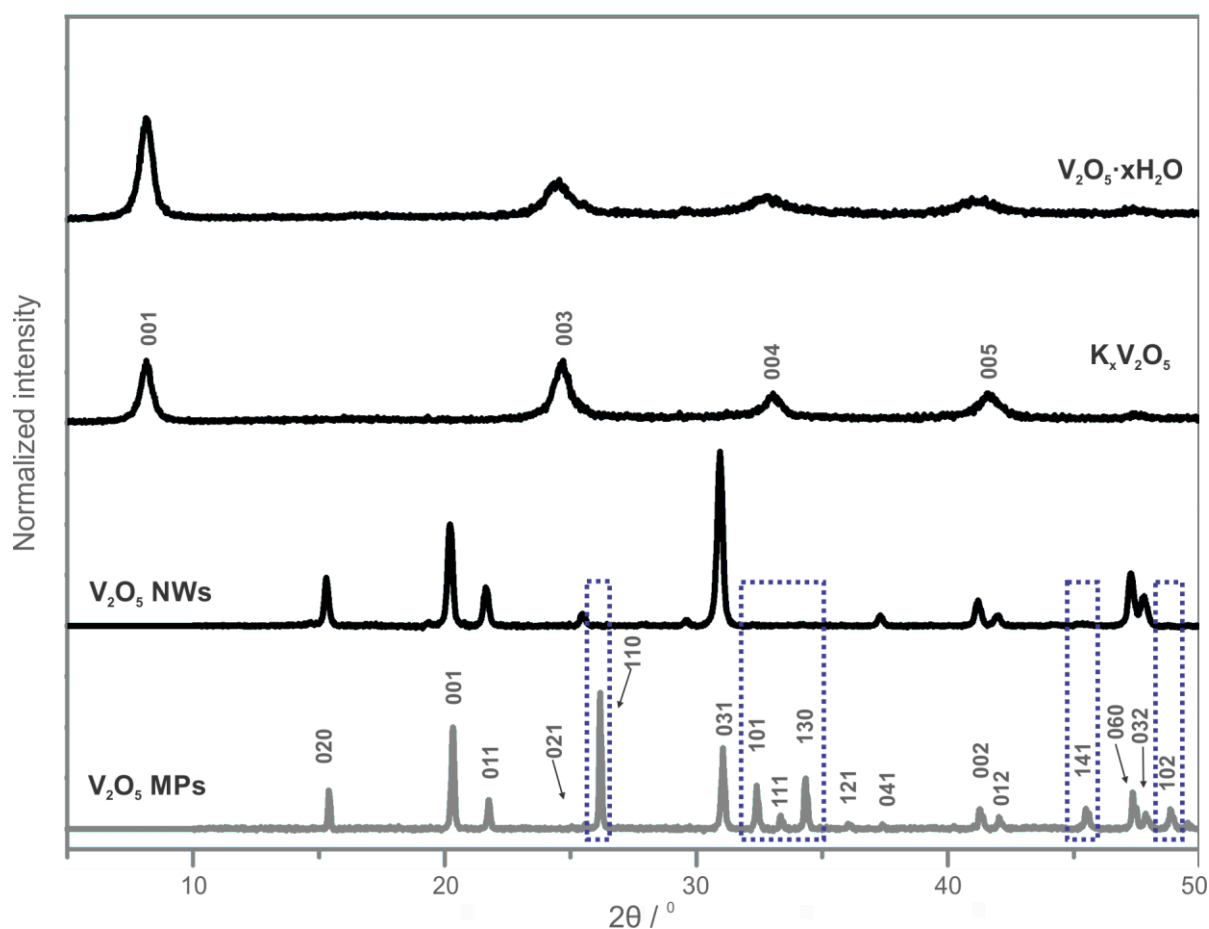


Figure 4.24: XRD patterns of the studied V_2O_5 powders.

The XRD patterns of both, $K_xV_2O_5$ and $V_2O_5 \cdot xH_2O$, were matched to the monoclinic unit cell crystal structure of vanadium pentoxide xerogel [91]. The molar ratio between monovalent K and V is 1:3 [94], so the phase composition was identified as beta- $K_{0.33}V_2O_5$. They exhibited only (00l) peaks, i.e., the peaks representing the (001), (003), (004) and (005) crystallographic directions (Fig. 4.24). Similar to the V_2O_5 NWs, these findings indicated a presence of preferential crystal orientation within both, $K_{0.33}V_2O_5$ and $V_2O_5 \cdot xH_2O$.

The orientation of the particles was further studied by the means of the SEM. The commercial V_2O_5 MPs had an irregular shape, without preferential orientation (Fig. 4.25a), i.e. they were isotropic microparticles. Their average size was 230 ± 25 nm. On the other hand, the V_2O_5 NWs particles exhibited nanostructured 1D morphology with a large aspect ratio (Fig. 4.25b). The average width of the particles was 40 ± 10 nm, while their average length was 25 ± 15 μ m. Thus, their aspect ratio was larger than 500. Similar to the V_2O_5 NWs particles, the $K_{0.33}V_2O_5$ and $V_2O_5 \cdot xH_2O$ particles exhibited 1D nanostructured morphology with average widths of 120 ± 40 μ m and 50 ± 20 μ m, respectively. However, they tended to bend, making the determination of their length difficult. From their SEM micrograph it was not possible to deduce the shape of nanoparticles (e.g., nanoribbons, nanowires, etc).

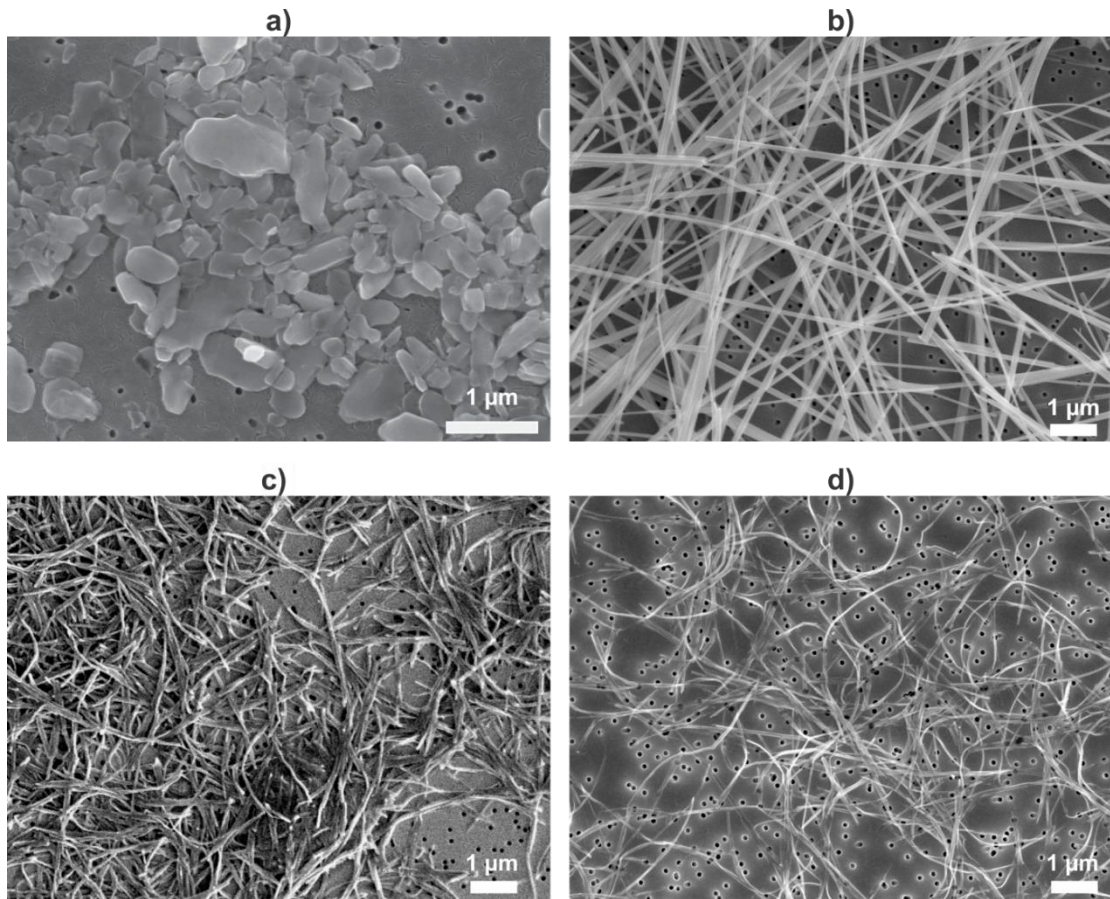


Figure 4.25: The SEM micrographs of the a) V_2O_5 MPs, b) V_2O_5 NWs, c) $K_xV_2O_5$ and d) $V_2O_5 \cdot xH_2O$ powders.

The shape and crystallinity of the 1D nanostructured particles was studied by the means of the TEM (Fig. 4.26). An individual 1D V_2O_5 NWs particle was a single crystal, as point diffraction was observed in the SAED pattern (Fig. 4.26a). The obtained distances in both the SEAD and the HR-TEM are complementary and I concluded that the growth

direction of the particles is (100) (Fig. 4.26a). The direction perpendicular to the particle growth was (011), proving that y - and z -directions are equivalent. From this I can state that the prepared V_2O_5 particles exhibited nanowire morphology, where individual nanowires are single-crystalline. Their large aspect ratio of the nanowires resulted in their horizontal fall on the XRD holder. Because of that, (h00) crystallographic planes are always in-plane with the holder and the XRD pattern exhibits only (0kl) diffractions (Fig. 4.24).

Similar to the V_2O_5 NWs particle, the SAED pattern proved that an individual 1D $K_{0.33}V_2O_5$ particle was a single crystal (Fig. 4.26b). The EDS analysis of the marked area (white circle, Fig. 4.26b) confirmed the presence of V, K and O, which was a proof of potassium intercalation in between the V_2O_5 lamellas. The obtained distances in the SEAD pattern confirmed that the growth direction of the particles was (100) while the perpendicular direction to the particle growth was (010) (Fig. 4.26b). This was a proof that particles preferentially grow in the x -direction. The particle growth was less expressed in y -direction and the least expressed in the z -direction. From this, I can state that the prepared $K_{0.33}V_2O_5$ particle exhibited a nanoribbon morphology. Because of that, the nanoribbons fall on the XRD holder in such a manner that the z -axis is perpendicular to the holder plane causing the (hk0) crystallographic planes to always be *in-plane* with the holder. Thus, the XRD pattern exhibits only (00l) diffractions (Fig. 4.24).

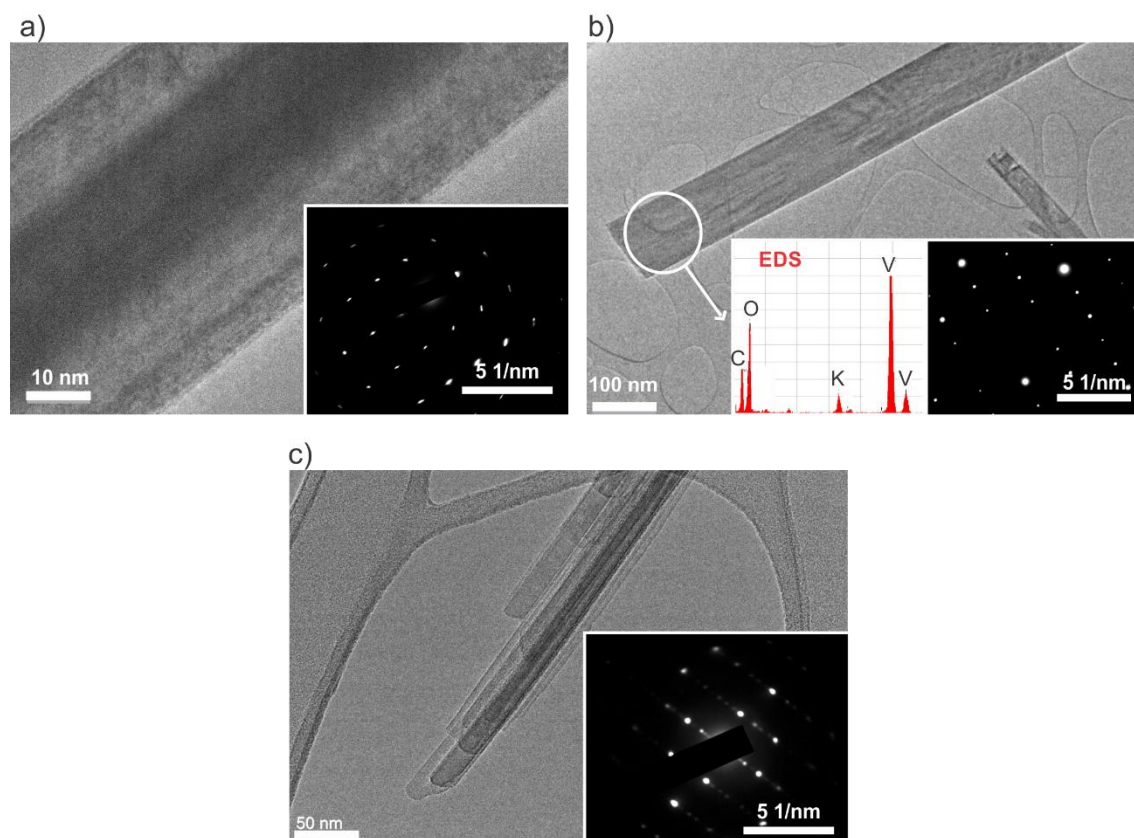


Figure 4.26: The TEM, SAED and EDS analysis of of the a) V_2O_5 NWs, b) $K_xV_2O_5$ and c) $V_2O_5 \cdot xH_2O$ particles.

Based on the SAED analysis, an individual $V_2O_5 \cdot xH_2O$ particle is grown in (100) direction which is similar to a $K_{0.33}V_2O_5$ particle (Fig. 4.26c). However, the point SAED pattern was disordered in the direction perpendicular to the direction of growth (Fig. 4.26c). The reason is turbostratic stacking of V_2O_5 lamellas in the z -direction [91]. Because $V_2O_5 \cdot xH_2O$ particles exhibited the same phase composition as $K_{0.33}V_2O_5$ particles (Fig.

4.24) and appeared similar upon the SEM observation (Fig. 4.25c, d), I concluded that they exhibited the nanoribbon morphology.

The amount of intercalated water within the $V_2O_5 \cdot xH_2O$ xerogel structure is variable and was depending on the applied temperature [142]. For example, the xerogel exhibited the molecular formula of $V_2O_5 \cdot 2.1H_2O$ when precipitated at room temperature or $V_2O_5 \cdot 1.1H_2O$ when thermally treated at $150^\circ C$ [142]. TGA analysis showed that $V_2O_5 \cdot xH_2O$ particles exhibited 3.3 % (w/w) mass loss in the temperature region from 220 – $360^\circ C$ (Fig. 4.27).

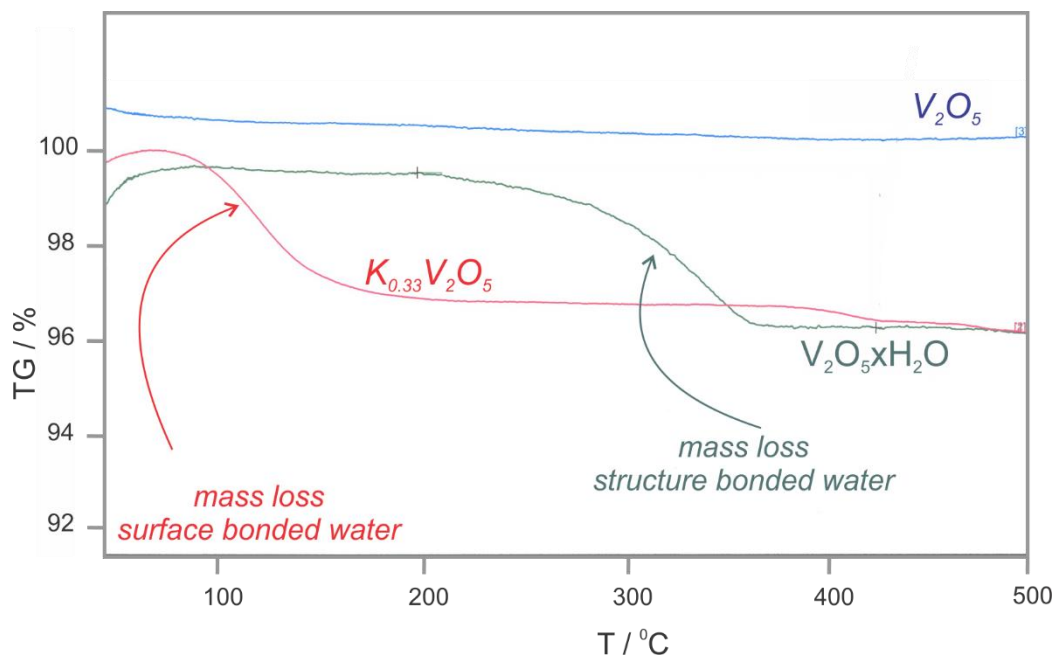


Figure 4.27: TGA mass loss analysis of the studied V_2O_5 powders.

Such loss was related to the release of structurally bonded water, which is situated between the V_2O_5 lamellas of a $V_2O_5 \cdot xH_2O$ nanoribbon. From the mass loss, I determined the structural formula to be $V_2O_5 \cdot 0.4H_2O$. In contrast, TGA analysis confirmed the absence of structurally bonded water within the structure of both, V_2O_5 NWs particles and $K_{0.33}V_2O_5$ nanoribbons. The TGA profile of the $K_{0.33}V_2O_5$ exhibited a mass loss in the temperature region from 80 – $150^\circ C$, which was attributed to the surface bonded water (Fig. 4.27).

4.2.2 The dissolution phenomena of the nanostructured V_2O_5 and antibacterial properties

The impacts of the dopants (K, H_2O) on the dissolution kinetics of nanostructured V_2O_5 as well as on its antibacterial properties were investigated.

The solubility assay showed that the initial rate of dissolution was greater for the V_2O_5 NWs than for the commercial V_2O_5 MPs in the PBS medium (Fig. 4.28). The vanadates' concentration in the V_2O_5 nanowire suspension was almost four times larger than the corresponding concentration in the commercial V_2O_5 suspension after 12 h ($t = 0.5$ days) of the exposure. The levels of the vanadates were equalized in both suspensions after a long ($t = 8$ days) exposure to the PBS medium (Fig. 4.28). Thus, there is a difference in the kinetics of dissolution, which was faster for the 1D nanoparticles than for the sub-micron particles. There is, however, no difference in the thermodynamics of the dissolution. The

nanoparticles dissolve faster than microparticles [102] due to the larger surface available for interaction with the solvent ($31 \text{ m}^2/\text{g}$ vs $4.5 \text{ m}^2/\text{g}$ in our study).

Both, $\text{K}_{0.33}\text{V}_2\text{O}_5$ and $\text{V}_2\text{O}_5 \cdot 0.4\text{H}_2\text{O}$, nanoribbons showed the 10 % lower levels of the released vanadates when compared to the V_2O_5 NWs (Fig. 4.28). The observed phenomena was not caused by the difference in the specific surface area of the powders because measured BET areas were $31 \text{ m}^2/\text{g}$ for the V_2O_5 NWs powder, $30 \text{ m}^2/\text{g}$ for the $\text{K}_{0.33}\text{V}_2\text{O}_5$ powder and $44 \text{ m}^2/\text{g}$ for the $\text{V}_2\text{O}_5 \cdot 0.4\text{H}_2\text{O}$ powder. Because of that, I concluded that it is related to the difference in structural characteristics of the particles.

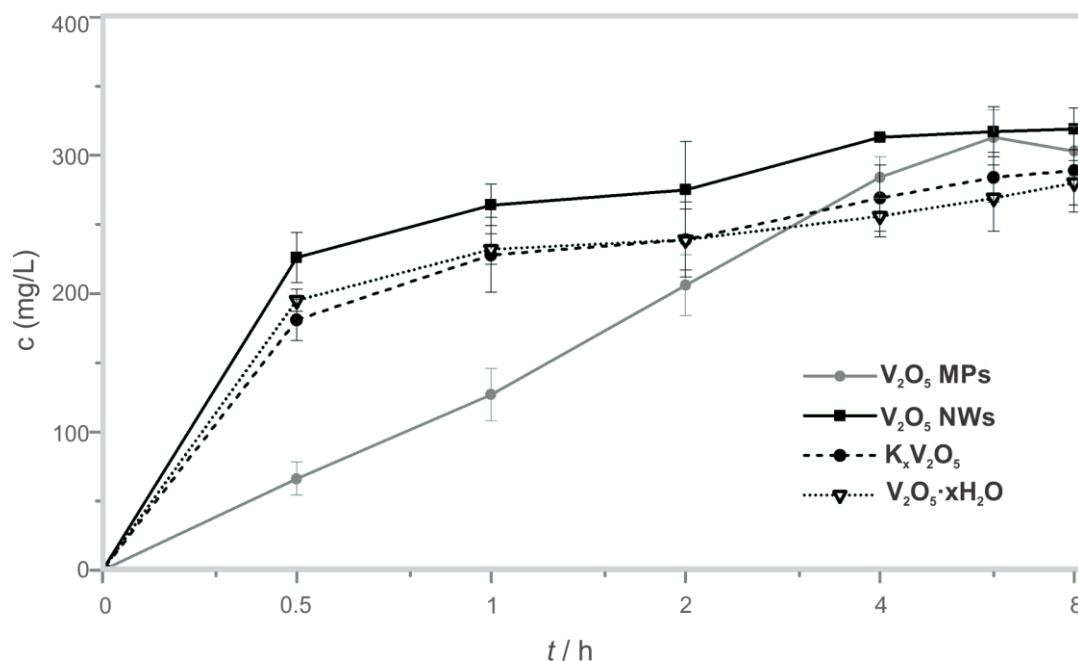


Figure 4.28: The vanadate release kinetics upon exposure of the studied V_2O_5 powders to the PBS medium.

In the further study of the V_2O_5 NWs dissolution phenomena their structural peculiarities after 1 day of exposure to the PBS were evaluated (Fig. 4.29). The XRD pattern of V_2O_5 nanowires was opposite to the pattern of nanowires before contact with the PBS (Fig. 4.29a). It exhibited peaks corresponding to the diffraction from the (110), (101), (111), (130), (121), (141) and (102) crystallographic planes (Fig. 4.29a). Thus, they were not placed exclusively parallel to the XRD holder, which indicated a decrease in the aspect ratio of the nanowires. The observation by the means of the SEM revealed that the V_2O_5 NWs were shorter after the exposure (Fig. 4.29b) when compared to the V_2O_5 NWs before the exposure (Fig. 4.25b). There was no observable change in the width of nanowires. The extent of the nanowires' length reduction is quantified and presented in a diagram (Fig. 4.29c). The average length of the nanowires is $25 \mu\text{m}$. After 1 day of exposure to PBS, their length is reduced by an order of magnitude, i.e., their average length is $2.2 \pm 1.7 \mu\text{m}$. A prolongation of the exposure to 4 and 8 days caused a further decrease in length to $2.0 \pm 1.9 \mu\text{m}$ and $1.8 \pm 1.8 \mu\text{m}$, respectively (Fig. 4.29c). The aspect ratio of the nanowires after the interaction with the PBS was reduced by an order of magnitude. Based on the obtained results, I concluded that the individual V_2O_5 nanowires dissolve via radial wire breakage.

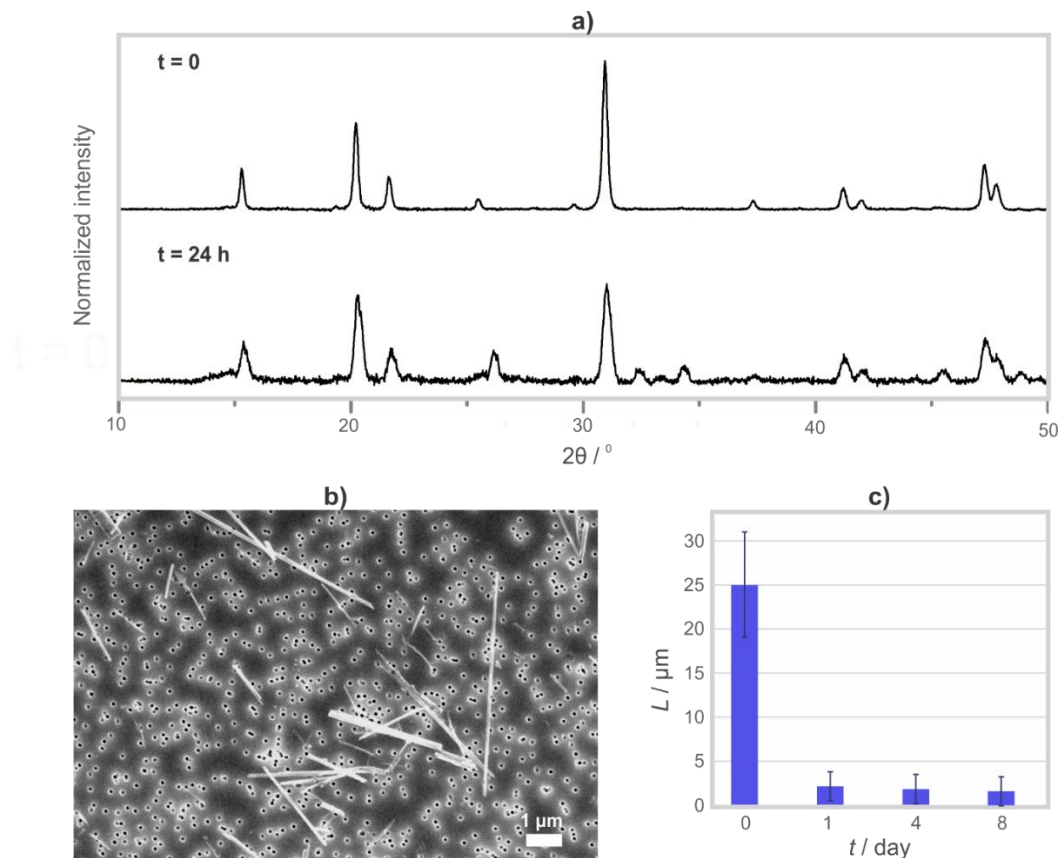


Figure 4.29: a) XRD spectrum of the V_2O_5 NWs before and after 24 h long exposure to the PBS medium; b) SEM micrograph of the V_2O_5 NWs after 24 h of exposure to the PBS medium; c) the average length of the V_2O_5 NWs as a function of the exposure time to the PBS medium.

The level of the eluted vanadates in all of the V_2O_5 suspensions was two orders of magnitude higher than bioactivity/cytotoxicity border, which confirmed the necessity of an efficient vanadate delivery system (Fig. 4.28).

I tested the lethal potential of the nanostructured V_2O_5 nanoparticles against bacteria via the biomimetic catalysis of the hydrogen-peroxide-to-hypochlorite transformation in the presence of Cl^- . Planktonic bacteria with 100% viability (according to a *BacLight live/dead* assay) were the starting point. (Fig. 4.30a). This starting viability was not significantly influenced when bacteria were exposed either i) to the vanadates, ii) to the V_2O_5 NWs or to iii) a combination of H_2O_2 and Cl^- (Fig. 4.30a). Such results indicated that neither the pure form of the V_2O_5 nor the released vanadates were able to harm the bacteria. However, the joint effect of the V_2O_5 and the chloride ions reduced the bacteria's viability by 75%. At the same time, the solution where the production of hypochlorite was expected (combined effect of V_2O_5 , Cl^- and H_2O_2) lowered the viability by just 25% (Fig. 4.30a), due to the increased solubility of the V_2O_5 in the presence of H_2O_2 [85].

In the phosphate buffer containing V_2O_5 and Cl^- , the number of eliminated *S. epidermidis* ($3.3 \cdot 10^6$ CFU/ml/g) is higher than the number of eliminated *E. coli* ($2.2 \cdot 10^6$ CFU/ml/g). Using this test, I confirmed the ability of the V_2O_5 NWs to cause the catalytically induced elimination of both the Gram-positive (*S. epidermidis*) and Gram-negative (*E. coli*) bacterial strains (Fig. 4.30c).

When the nanoribbons (either $\text{K}_{0.33}\text{V}_2\text{O}_5$ or $\text{V}_2\text{O}_5 \cdot 0.4\text{H}_2\text{O}$) were compared to the V_2O_5 NWs, the strong drop in the bactericidal potential was observed (Fig. 4.30b). $\text{K}_{0.33}\text{V}_2\text{O}_5$

nanoribbons reduced bacteria viability for just 35 % while $\text{V}_2\text{O}_5 \cdot 0.4\text{H}_2\text{O}$ nanoribbons eliminated just 25 % of bacteria through 8 h long exposure. Even though the dissolution of the nanoribbons was reduced for 10% when compared to the V_2O_5 NWs (Fig. 4.28), the drop in antibacterial activity was almost 3-fold (Fig. 4.30b). Because of that, only V_2O_5 NWs were utilized in preparation of V_2O_5 /Polymer coatings among the synthesized nanostructured V_2O_5 particles.

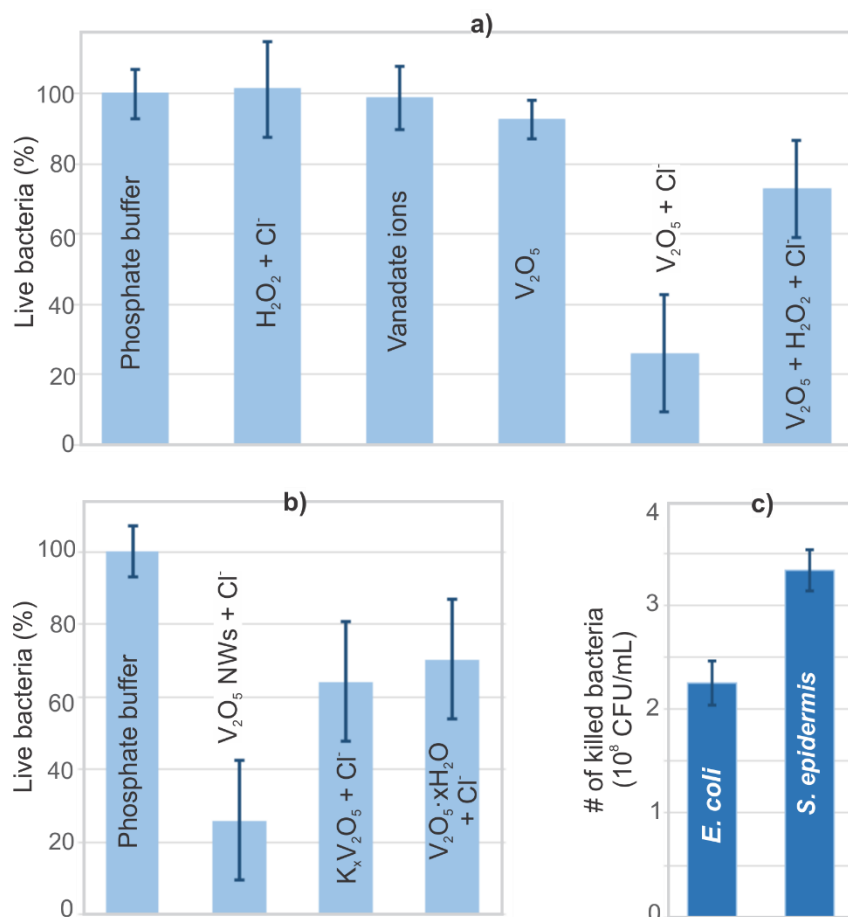


Figure 4.30: a), b) Percentage of viable *E. coli* after 8 h of exposure to the studied V_2O_5 powders; c) Number of killed *E. coli* and *S. epidermidis* within the 8 h of bacteria exposure to 1 g/ml suspension of the V_2O_5 .

4.3 Magnesium-Oxide/Polymer Composite Coatings

The MgO/polymer composite coatings were prepared by the means of a solvent casting method. During the process, the correlation between MgO 900/polymer physicochemical properties, magnesium elution kinetics and antibacterial properties was studied. At the end, the hemocompatibility of the prepared coatings was evaluated. The MgO NPs/polymer composites were used as a reference.

4.3.1 Physicochemical properties of MgO/polymer composites

The ability of different polymers to control the solubility of embedded nano-textured MgO 900 microrods was evaluated. The analysis included the observation of composite coatings' surface morphologies and assessment of the interphase MgO/polymer interactions.

The inspection of the MgO/polymer composites' surface morphology was performed by the means of the SEM. The surface of the MgO NPs/PLGA composite was rough. The small and large MgO NPs agglomerates were observed (Fig. 4.31a). The extent of the MgO particles' coverage with the PLGA matrix varied. Some particles were almost completely covered with the matrix (marked with a dashed rectangle), while others were just slightly immersed inside the matrix (marked with a solid rectangle) (Fig. 4.31a). The MgO 900 microrods' nano-texturing was hidden by the PLGA matrix, which indicated their complete coverage (Fig. 4.31b) within the MgO 900/PLGA composite coating. The microrods were dispersed along the matrix and were placed almost *in plane* with the coating's surface.

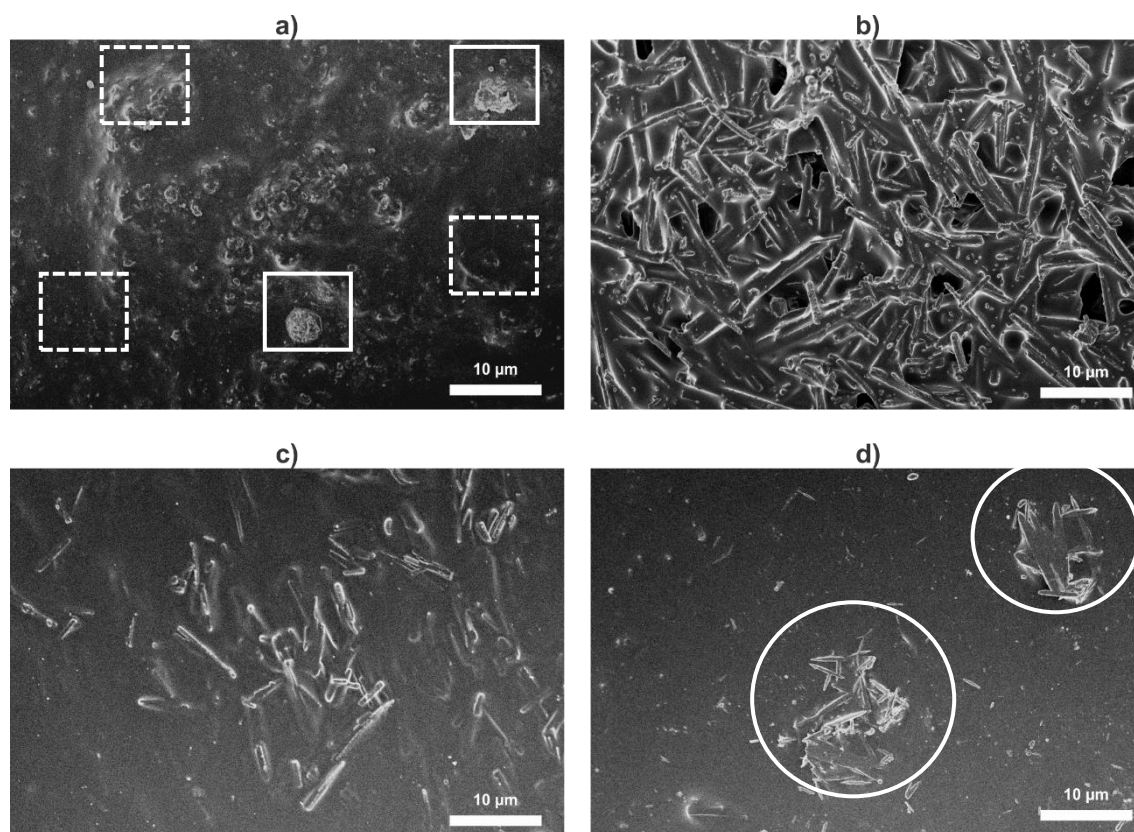


Figure 4.31: The SEM imaging of a) MgO NPs/PLGA, b) MgO 900/PLGA, c) MgO 900/PLA and d) MgO 900/PCL composite coatings. The MgO mass fraction in the coatings was 25 %.

Similar to the MgO 900/PLGA composite coating, the microrods were completely covered by the PLA and PCL matrix (Fig. 4.31c, d). However, they were placed along the PLA matrix in the less-well dispersed manner compared to the PLGA matrix (Fig. 4.31b, c). In the case of the MgO/PLA composite coatings, the microrods were assembled in clusters at the surface of MgO/PCL matrix (Fig. 4.31d, white circles). These results indicated the difference in the interphase interaction among different MgO 900/polymer composites.

The interface interactions within MgO 900/polymer were further studied by the means of the ATR-IR spectroscopy. The IR absorptions of MgO in the ATR mode were weak when compared to the polymers (Fig. 4.32). The ATR-IR spectrum of the MgO (Fig. 4.32) contained absorption maxima assigned to Mg-O vibrations. The first one was at 860 cm^{-1} while the second one was just partially observed because its maximum value was below 650 cm^{-1} [143]. In addition, IR spectrum of MgO 900 indicated the presence of surface bonded carbon dioxide (absorption maximum at 1430 cm^{-1}) and water (absorption maximum at 1650 cm^{-1}) [143].

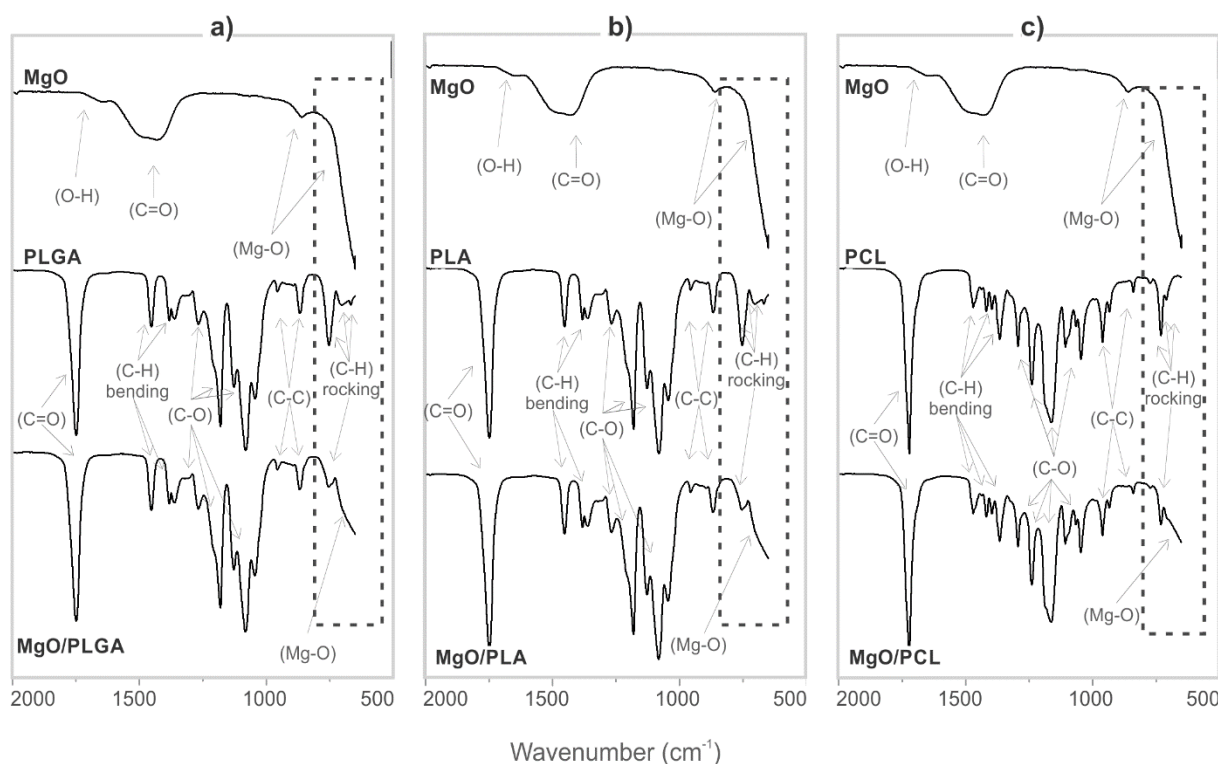


Figure 4.32: The ATR-IR spectra of the a) MgO 900, PLGA and MgO/PLGA; b) MgO 900, PLA and MgO/PLA and c) MgO 900, PCL and MgO/PCL. The MgO mass fraction in the coatings was 25 %.

The ATR-IR spectrum of the PLGA (Fig. 4.32a) showed the presence of its characteristic poly-ester absorption bands. The absorption bands at 668 , 712 and 753 cm^{-1} correspond to the C-H rocking vibrations [144], [145]. The sharp absorption maximum at 1746 cm^{-1} is assigned to the vibration of the carbonyl group (C=O) [144], [145]. The bands at 1271 , 1162 , 1130 , 1084 and 1050 cm^{-1} are assigned to the C-O stretching bond vibration, while the bands at 1452 , 1432 , 1393 , 1384 and 1366 cm^{-1} belong to the C-H bending vibrations [144], [145]. Furthermore, the absorption maxima at 956 , 922 , 891 , 868 and 846 cm^{-1} correspond to the C-C stretching vibrations [144], [145]. Similar to the PLGA, the PLA

and PCL also exhibited the poly-ester characteristic bands. The values were summarized in Table 4.1.

Table 4.1: Characteristic absorption bands of the used polyesters [144]–[146].

Vibration bands	C=O	C-H bending	C-O	C-C	C-H rocking
PLGA	1746	1452, 1432, 1393, 1384, 1366 cm^{-1}	1271, 1162, 1130, 1084, 1050 cm^{-1}	956, 922, 891, 868, 846 cm^{-1}	668, 712 and 753 cm^{-1}
PLA	1730	1452, 1432, 1393, 1384, 1366 cm^{-1}	1271, 1162, 1130, 1084, 1050 cm^{-1}	956, 922, 891, 868, 846 cm^{-1}	668, 712 and 753 cm^{-1}
PCL	1720	1420, 1396, 1396, 1367 cm^{-1}	1294, 1239, 1162, 1105, 1050 cm^{-1}	961, 932, 838 cm^{-1}	707, 728 and 751 cm^{-1}

The ATR-IR spectrum of the MgO 900/PLGA exhibited the presence of both Mg-O vibrations and all of the corresponding PLGA vibration bands (Fig. 4.32a). The IR absorption bands related to the impurities at the surface of MgO were no longer observed. The other difference is the reduction in the intensity of the C-H rocking vibrations. When intensities of the absorption maximum at 753 cm^{-1} were compared, I observed 80 % reduction in the intensity in the IR spectra of the MgO 900/PLGA when compared to the IR spectra of the PLGA (Fig. 4.32a). Similarly, the ATR-IR spectra of the MgO 900/PLA and PCL exhibited the presence of Mg-O vibrations together with all of the corresponding PLA and PCL vibration bands, respectively (Fig. 4.32b, c). Again, the reduction in the intensity of the C-H rocking vibrations was observed when IR spectra of MgO 900/polymer composites were compared to the IR spectra of pure polymers. In the case of MgO 900/PLA composite this reduction was equal to the 70 %, while in the case of MgO 900/PCL this reduction was equal to the 55 % (Fig. 4.32b, c).

The reduction in the IR intensity of C-H rocking vibrations was the largest in the case of PLGA matrix and the lowest in the case of PLA matrix. These results indicated that the studied polyesters interact via their C-H bonds with the MgO 900 particles. Based on the obtained results, the interphase interactions were the strongest in the case of MgO/PLGA and the weakest in the case of MgO/PCL.

4.3.2 The dissolution phenomena of the MgO/polymer composite coatings

In this section, the role of the particle morphology (MgO NPs vs MgO 900 microrods) and polymer type (PLGA, PLA, PCL) on the elution kinetics of the magnesium from the MgO 900/polymer composites to the physiological solution was investigated. The analysis included the observation of composite coatings' surface morphologies after the immersion in the physiological solution and quantification of elution kinetics.

The immersion of the MgO/polymer coatings into the physiological solution for two hours caused changes in the morphology of the coatings. The surface of MgO NPs/PLGA coating contained holes. The majority of them were below 200 nm in size, which was related to the degradation of the PLGA matrix (Fig. 4. 33a). However, some of them were several mm in size and were formed when large agglomerates of the MgO NPs eroded from the surface towards the surrounding medium (Fig. 4. 33a, enlarged area). Thus, exposure of the

MgO NPs/PLGA composite coatings to the physiological medium caused erosion of the agglomerates, i.e. PLGA matrix was not able to retain MgO NPs at its surface.

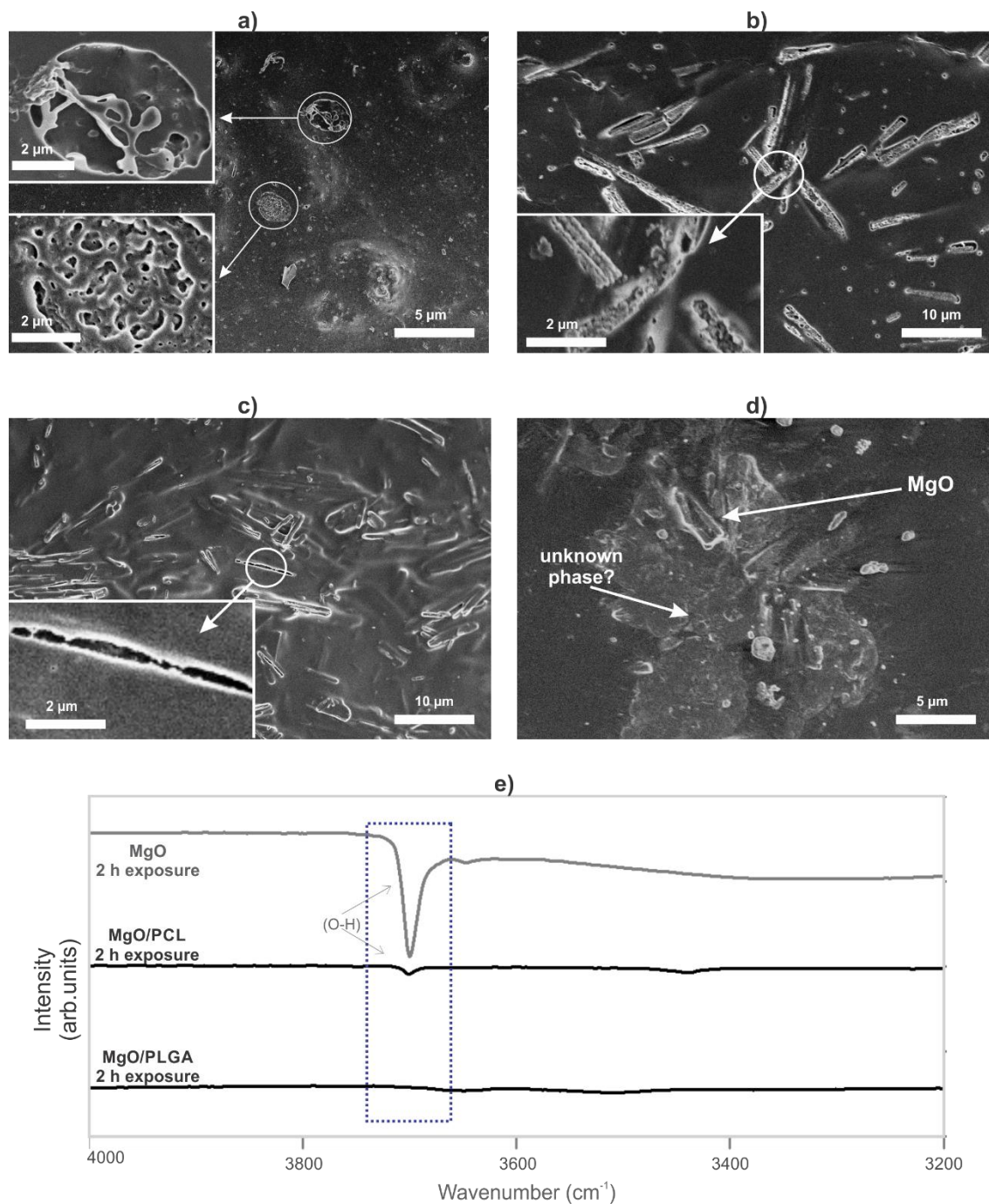


Figure 4.33: The SEM images of a) MgO NPs/PLGA, b) MgO 900/PLGA, c) MgO 900/PLA and d) MgO 900/PCL composite coatings after 2 h of exposure to the physiological solution; e) The IR spectra of either MgO 900, MgO 900/PCL or MgO 900/PLGA after 2 h of exposure to the physiological solution. The MgO mass fraction in the coatings was 25 %.

The surface of the MgO 900/PLGA coating also contained two types of holes. Small holes, up to 300 nm in size were a consequence of PLGA degradation. The larger ones had the shape of microrods. Within them, the MgO 900 particles were situated (Fig. 4. 33b)

and exhibited nano-texturing of their surface (Fig. 4. 33b, enlarged area), opposite to the coating before exposure (Fig. 4. 31b). Such observation implies exposure of the MgO microrods to the surrounding physiological solution while, at the same time, PLGA matrix retained them on its surface.

The applied PLA and PCL degrade slower than the PLGA in the physiological conditions according to the supplier certificates. Because of that, the surface of MgO 900/PLA was free from the small degradation-caused holes (Fig. 4.33c). A few holes in the shape of the microrods were observed, but within them there were no situated MgO microrods (Fig. 4.33c enlarged area). MgO microrods on the surface were still completely covered with the PLA matrix, as their nano-textured surface was not detected. These results indicated that PLA matrix made the MgO surface less accessible to the surrounding medium due to their slower degradation kinetics compared to the PLGA. At the same time, the microrods, which got exposed, were not retained at the PLA surface.

Similar as at the surface of the MgO 900/PLA, the nano-textured MgO microrods were still covered with the PCL matrix at the surface of MgO/PCL composite and there were no visible degradation-related holes at the surface (Fig. 4. 33d). Apart from the covered MgO microrods, an unknown phase was observed at the surface of the MgO/PCL coatings (Fig. 4. 33d). The determination of its structure was performed by the means of the ATR IR spectroscopy. As a consequence of dissolution and phase transformation upon the exposure to the physiological solution, the ATR-IR spectrum of the MgO 900 microrods was changed. I observed the evolution of the sharp absorption maxima at 3700 cm^{-1} (Fig. 4.33e), which was not observed at the ATR-IR spectra of the as-synthesized MgO 900 (Fig. 4.7b). During the exposure, oxide to hydroxide transformation occurred. Thus, this vibration band represents O-H vibration of the formed hydroxide phase. When MgO/PLGA was exposed to the physiological solution for 2 h, this vibration band was not observed (Fig. 4.33e). However, in the case of the MgO/PCL, small vibration band representing hydroxide phase was identified (Fig. 4.33e) which led to the conclusion that the unknown phase (Fig. 4. 33d) is a magnesium hydroxide. These results emphasized the ability of the PLGA to prevent dissolution and phase transformation of the microrods in MgO 900/ PLGA composite within first 2 h of exposure.

The magnesium release kinetics from the prepared MgO/polymer coatings were quantified. Firstly, the impact of particle morphology was evaluated. When MgO NPs/PLGA composite was exposed to the physiological solution, more than 35 % of MgO was released within the first day of the exposure (Fig. 4.34), i.e. strong initial burst effect was observed. After the first day, the release was stabilized in the range from 2-6 % of the released MgO NPs. After 6 days of exposure, a total of 55 % percent of the agglomerated nanoparticles was released from the MgO NPs/PLGA to the surrounding medium. After the release, they were either dissolved or transformed to the magnesium hydroxide.

In contrast, the increase in magnesium ions concentration in the physiological solution above the MgO 900/PLGA coatings was more controlled. During the first day, 12 % of the MgO 900 particles were released to the medium which was a three-fold decrease when compared to the MgO NPs in the PLGA matrix (Fig. 4.34). These results were explained by the tendency of PLGA to retain MgO 900 microrods at its surface and to release MgO NPs, as previously observed at the surface of the composite coatings (Fig. 4.34a, b). After the first day, the daily elution kinetics was stabilized in the range from 4-8 % per day, which is more than in the case of MgO NPs. After 6 days of exposure, 38 % of the MgO 900 was released from the coating (Fig. 4.34). Such results clearly demonstrated a more controlled release of MgO 900 from the PLGA matrix compared to the release of MgO NPs, i.e. application of 1D particles with reduced agglomeration instead of commercial agglomerated isotropic particles provided a more controllable release kinetics from the polymer matrix.

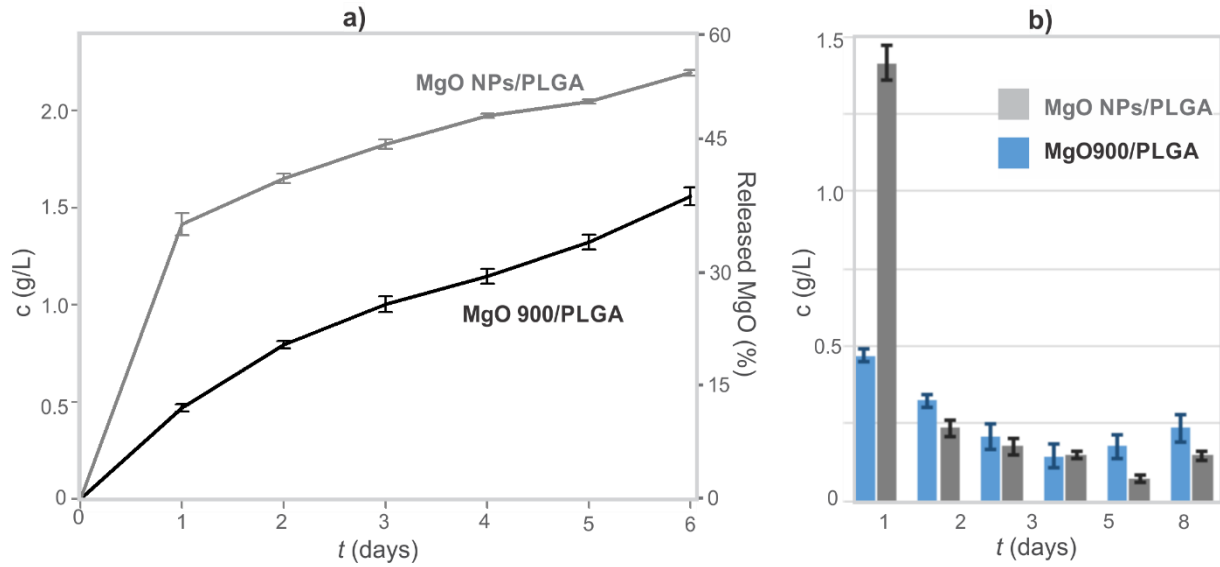


Figure 4.34: a) The cumulative and b) the daily release kinetics of the magnesium when the MgO NPS/PLGA and MgO 900 /PLGA composite coating were exposed to the physiological solution.

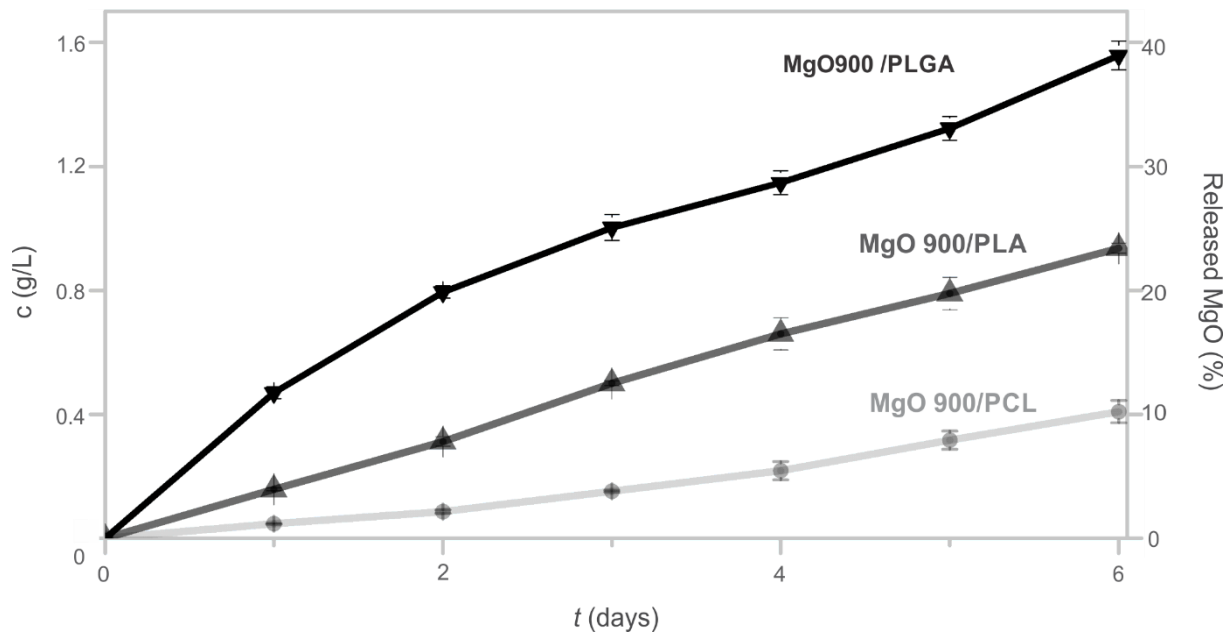


Figure 4.35: The cumulative magnesium elution kinetics from different MgO 900/polymer composites, presented as either an increase in magnesium concentration or percentage of released MgO from polymer matrix.

The magnesium release kinetics from the MgO 900/PLA and MgO 900/PCL composite coatings followed similar trends as in the case of the MgO 900/PLGA composite. The initial burst effect was suppressed in all three cases, especially in the MgO/PCL composite coatings where only 1.5 % of MgO was released to the medium (Fig. 4.35). After six days of exposure, 23 % and 10 % were released from the MgO 900/PLA and MgO 900/PCL composite coatings, respectively, which is much lower than 38 % of released MgO 900 from the PLGA matrix. This trends follow the polarity of the matrixes. The most polar matrix (PLGA) exhibited the highest release of the nano-textured MgO microrods.

However, the SEM observation of the morphology indicated that MgO microrods were largely covered by the polymer matrix within MgO/PLA and MgO/PCL composites (Fig. 4.33c, d). Once exposed, they were immediately released to the surrounding medium. On the other hand, the exposed MgO 900 microrods at the surface of PLGA matrix were retained for a certain period of time (Fig. 4.33c). Taking into account that MgO exhibits a contact-based mechanism of activity, I concluded that optimal coating protection would be in the case when MgO particles are exposed to the surrounding medium but still situated at the surface. As a result, the MgO 900/PLGA composite coating was chosen as the most appropriate for further testing with biological systems among three MgO 900/polymer coatings.

4.3.3 The antibacterial properties of the MgO/polymer composite coatings and their hemocompatibility

The correlation between the magnesium release from the prepared MgO/PLGA composites and their bactericidal potential was a matter of interest. Both, MgO NPs/PLGA and MgO 900/PLGA were subjected to pre-treatment, which included exposure to the physiological solution for either one (one-day old coatings) or three (three-day old coatings) days, respectively. After such exposure, the coatings were washed and bacteria suspension was added. The lethal kinetics of the coatings against *E. coli* was measured. The one-day old MgO NPs/PLGA and MgO 900/PLGA coatings eliminated the *E. coli* in a steady manner (Fig. 4.36a). However, MgO 900/PLGA coatings were more efficient as they eliminated 75 % of all bacteria within 8 h of exposure, compared to 58 % lethal efficiency of MgO NPs/PLGA (Fig. 4.36a).

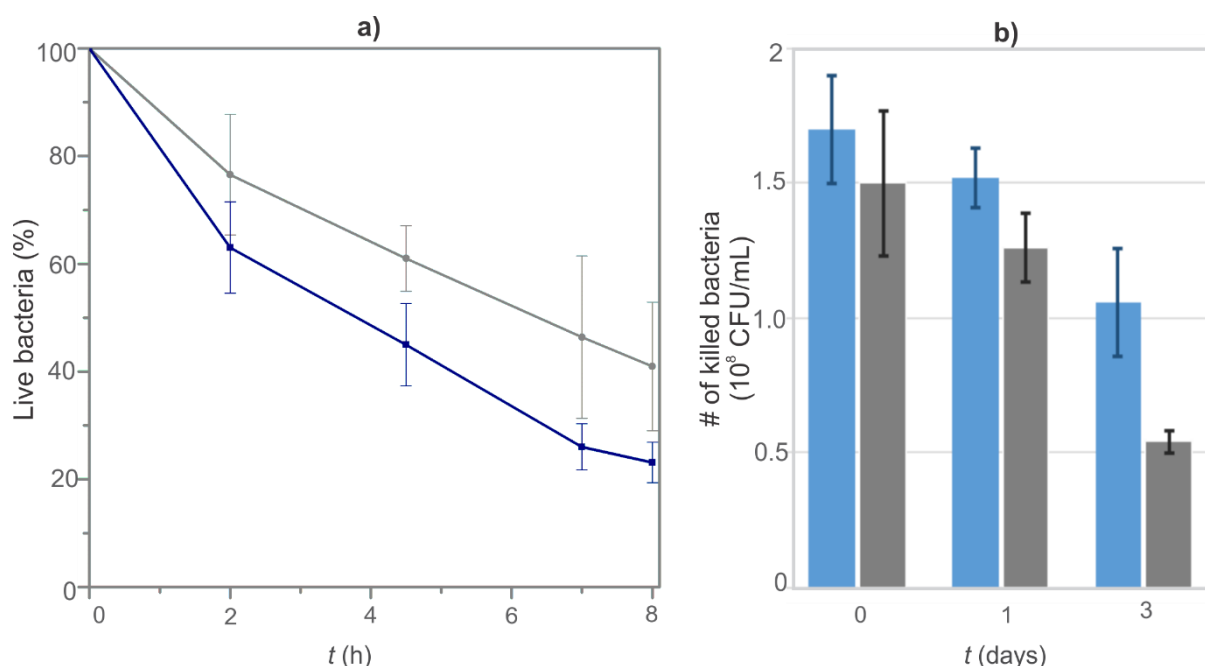


Figure 4.36: a) The percentage of viable *E. coli* as a function of the exposure time to MgO/PLGA composite coatings; b) the number of killed *E. coli* within 8 h of exposure to the MgO/PLGA composite coatings, previously exposed to physiological solution for different time intervals.

The lethal efficiency of MgO/PLGA coatings was decreasing with an increase in the length of pre-treatment. It dropped from 85 % (as-prepared MgO 900/PLGA coating) over 75 % (one-day old MgO 900/PLGA coating) to 53 % (three-day old MgO 900/PLGA coating). Thus, over three days of exposure the lethal potential dropped for 32 % (Fig. 4.36b). The drop in lethal efficiency was more pronounced for MgO NPs/PLGA coatings, as it was reduced from 75 % (as-prepared coating) to 27 % (the three-days old coating) (Fig. 4.36b). Thus, the lethal efficiency of the MgO NPs/PLGA coatings exhibited a three-fold drop over the three days of exposure.

Such results confirmed that nano-textured MgO 900 microrods provide more sustained antibacterial protection of the composite coating surface than the MgO NPs. I related observed phenomena to the ability of PLGA to better retain the 1D microrods instead of the agglomerated nanoparticles on its surface.

The ability of the MgO 900 microrods within the PLGA matrix to prevent *S. epidermidis* colonization of the composite surface was evaluated. Both, PLGA and MgO 900/PLGA composite coatings, were incubated in the suspension containing *S. epidermidis*. After 24 h of incubation, the metabolic activity of the bacteria at the surface of the coatings was determined by the means of fluorescent spectroscopy. The strong fluorescent signal was detected in the case of the PLGA coatings while in the case of the MgO/900 the fluorescence signal was not significantly different from the blank solution without bacteria (Fig. 4.37a), which confirmed the strong metabolic activity of the bacteria at the surface of the PLGA coating and the absence of metabolic activity at the surface of MgO 900/PLGA composite coating.

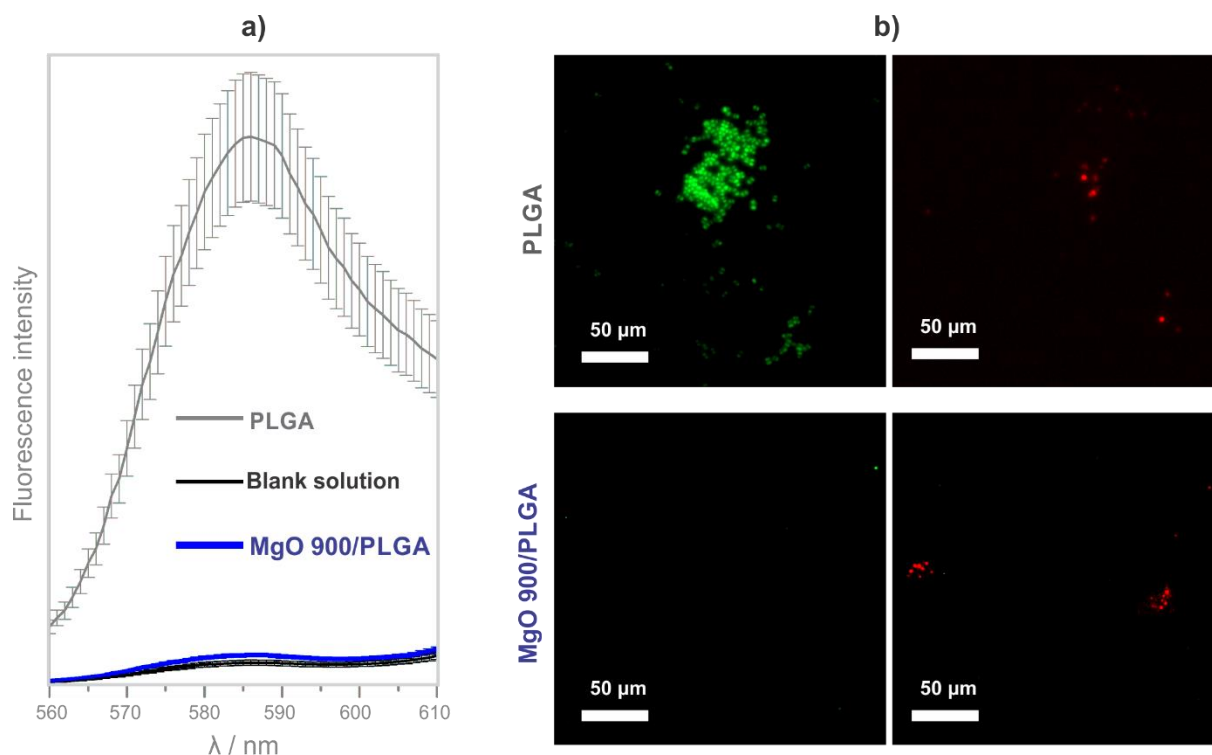


Figure 4.37: a) The metabolic activity of attached *S. epidermidis* at the surface of either PLGA or MgO/PLGA coatings determined by the extent of resazurin-to-resorufin transformation. The excitation wavelength was 530 nm; b) the fluorescent Backlight live/dead imaging of the attached *S. epidermidis* at the surface of either PLGA or MgO/PLGA coatings after 24 h long incubation at 37 °C.

The viability of the colonized bacteria was further studied by the means of *live/dead* BacLight fluorescent assay. The clusters of bacteria at the surface of PLGA emitted a strong green signal, while the red signal was scarcely occurring (Fig. 4.37b). In contrast, absence of green signal and dominance of red signal was emitted from the surface of the MgO 900/PLGA coating. In addition, the abundance of *S. epidermidis* clusters on the surface of MgO/PLGA coating was much lower than on the surface of the PLGA coating (Fig. 4.37b). These results proved the presence of predominantly viable bacteria on the surface of the PLGA coating and the presence of just non-viable bacteria at the surface of the MgO 900/PLGA composite coating.

When a biomaterial is implanted within the body, the host body will first undergo a universal response to the materials which is an extension of the mammalian response following tissue injury. In the first step, it is exposed to blood and interstitial fluids which leads to a formation of transient surface matrix within seconds after exposure [19]. Whole sheep blood constitute up to 99 % of the red blood cells (RBCs) [134] and are likely to be the first cells in the contact with the surface of the implanted biomaterial. Because of that, structural changes of the RBCs during their incubation with the prepared coatings were further investigated.

The hemolysis of the RBCs causes disruption of their membrane and release of haemoglobin to the surrounding medium [134]. As a result, the solution becomes red, which allows a spectrophotometric measurement of the absorbance. In this study, absorbance at 415 nm was proportional to the amount of hemolysed cells and a calibration curve was obtained (Fig. 4.38a).

After a 24 h long incubation of the prepared coatings with the blood, the amount of released haemoglobin was measured. Using the previously prepared calibration curve, the percentage of hemolysed RBCs was determined. The results indicated that exposure of the PLGA coating, as well as MgO 900/PLGA coatings with 10 % and 1 % of MgO, to the blood solution did not cause hemolysis of RBCs. In contrast, 15 % of RBCs were hemolysed when the MgO 900/PLGA coating with 25 % of MgO (Fig. 4.38b) while 80 % of RBCs were hemolysed when exposed to MgO 900 particles in solution for 24 h. After the exposure to either the PLGA or the MgO 900 (10%)/PLGA coating, RBCs were observed by the means of optical microscopy. I did not observe any traces of morphology changes nor the presence of “ghost cells” in both cases (Fig. 4.38c). These results indicated that the composite coatings containing 10% or less of MgO did not cause mechanical damage of the RBCs and release of haemoglobin.

The determination of the extent of RBCs hemolysis provided information about the average impact of the prepared coatings on the blood dilution in the PBS. However, it is desirable to evaluate impact of the coatings to the RBCs in the vicinity of a coating surface. For that purpose, I observed the morphology of the cells attached at the coatings surface by the means of SEM. A normal RBC assumes a biconcave discoid shape [147]. The RBCs at the surface of the PLGA coating exhibited modified membrane morphology, as series of crenated shapes, called echinocytes, were observed (Fig. 4.38d) [147]. The measurement of the supernatant pH after the PLGA coating was incubated in blood suspension in PBS was reduced from 7.4 (PBS) to 7.25. Such effect of such small change in the pH was more pronounced in the vicinity of the coating, which might explain the evolution of the crenated shapes at the surface of the RBCs. Introduction of 10 % of MgO within the PLGA matrix resulted in the reduction of the number of RBCs with crenated shapes attached at the coating surface (Fig. 4.38d). At the same time, the supernatant pH after the MgO 900/PLGA coating was incubated in blood suspension in PBS was 7.4, which is the same as in the PBS. Therefore, the introduction of MgO within the PLGA matrix reduced its negative effect to the RBCs.

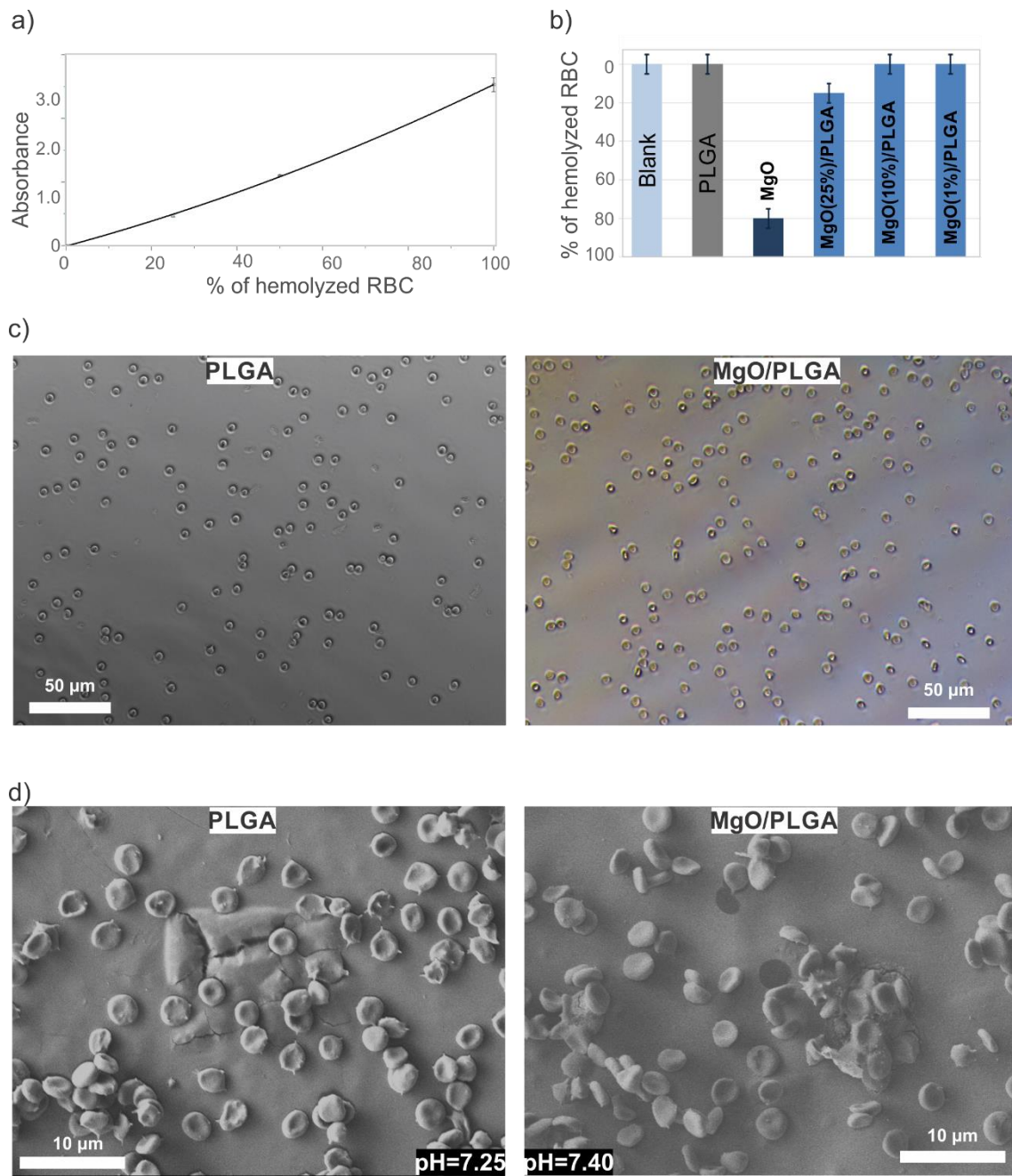


Figure 4.38: a) The RBCs' hemolysis calibration curve; b) the % of hemolysed RBCs after 24 long exposure to the various composite coatings; the morphology of the RBCs at the surface of either PLGA or MgO(10%)/PLGA coatings observed by the means of c) optical microscopy and d) SEM. The pH of the RBCs suspension after the exposure is provided on the SEM images.

4.4 Vanadium-Pentoxide/Polymer Composite Coatings

The V_2O_5 /polymer composite coatings were prepared by means of a solvent casting method. During the process, the correlation between the prepared V_2O_5 /polymer physicochemical properties, vanadate elution kinetics and antibacterial properties was studied. At the end, the hemocompatibility of the prepared coatings was evaluated.

4.4.1 Physicochemical properties of V_2O_5 /polymer composites

The ability of different polymers to control the solubility of embedded V_2O_5 NWs was evaluated. The analysis included the observation of composite coatings' surface morphologies and assessment of the interphase V_2O_5 /polymer interactions.

The inspection of the V_2O_5 /polymer composites' surface morphology was performed by the means of the SEM. The composite based on the PLGA matrix exhibited embedded V_2O_5 MPs particles on the surface (Fig. 4.39a). The extent of the V_2O_5 particles' coverage with the polymer matrix varied. Some particles were almost completely covered with the PLGA matrix (marked with a dashed rectangle), while others were just slightly immersed inside the matrix (marked with a solid rectangle). In comparison to the V_2O_5 MPs, the coverage of the V_2O_5 nanowires by the PLGA matrix was better (Fig. 4.39c), as just small portions of the individual nanowires were exposed to the environment. The nanowires were dispersed along the matrix and lay almost *in plane* with the surface. We observed a similar distribution of nanowires at the surface of the V_2O_5 NWs/PLA-and V_2O_5 NWs/chitosan (Fig. 4.39e, k). In contrast, at the surface of V_2O_5 NWs/PS composites bunches of agglomerated nanowires were identified (Fig. 4.39g).

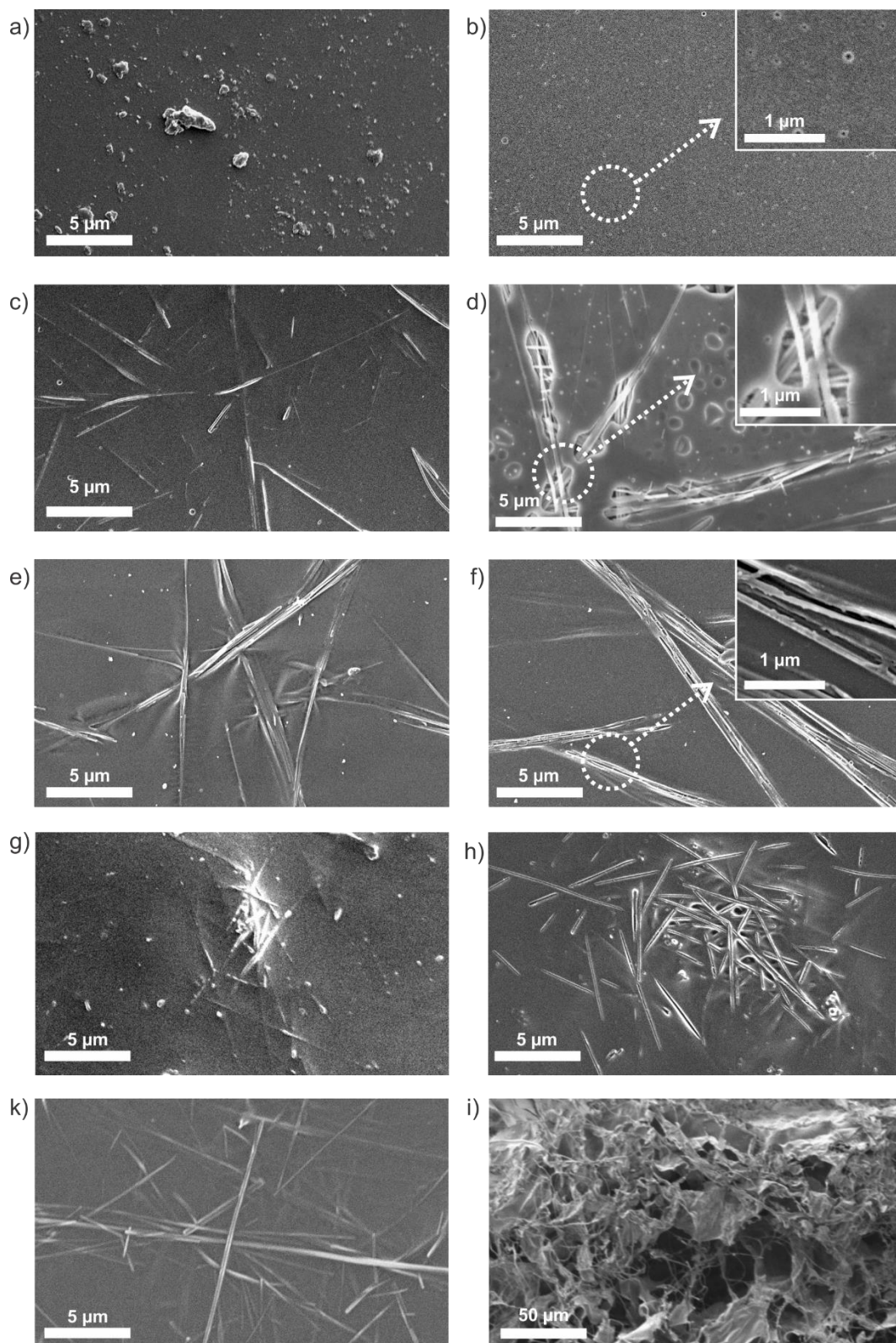


Figure 4.39: SEM micrographs of the surface of commercial V_2O_5 /PLGA composite a) before and b) after exposure to PBS; V_2O_5 nanowires/PLGA c) before and d) after exposure to PBS; V_2O_5 nanowires/PLA e) before and f) after exposure to PBS; V_2O_5 nanowires/PS g) before and h) after exposure to PBS; V_2O_5 nanowires/Chitosan k) before and i) after exposure to PBS. Coatings were exposed to the PBS for 1 day.

The nature of the interphase interactions inside the V_2O_5 NWs/polymer composites was studied using the FT-IR technique. The ATR-IR spectrum of the V_2O_5 nanowires (Fig. 4.40a) consists of two absorption maxima. The sharp one, at 1012 cm^{-1} , is assigned to the vibration modes of the $V=O_A$ bond. This oxygen is bonded to a single vanadium atom. The broad band at 858 cm^{-1} corresponds to the vibrations of the $V-O_B-V$ structure. The O_B is bonded to two vanadium atoms and is a bridging atom between two corner-sharing octahedra [148], [149]. The ATR-IR spectrum of PS (Fig. 4.40a) showed the presence of its characteristic bands. Those in the range from 2700 to 3100 cm^{-1} are assigned to the C-H stretching vibrations. The absorption maxima in the range from 1400 to 1600 cm^{-1} correspond to the C=C stretching vibration of the styrene aromatic rings. The bands in the range from 1000 to 1275 cm^{-1} belong to the in-plane C-H bending, while the absorption maxima in the range from 600 to 900 cm^{-1} belong to the out-of-plane C-H bending [150]. The ATR-FTIR spectrum of the V_2O_5 /PS composite is a combination of the individual V_2O_5 and PS IR spectra, respectively (Fig. 4.40a). In the regions where individual bands are overlapping, a convolution of peaks is observed (e.g., the band of PS at 854 cm^{-1} convolutes with the V_2O_5 band at 858 cm^{-1} , as indicated).

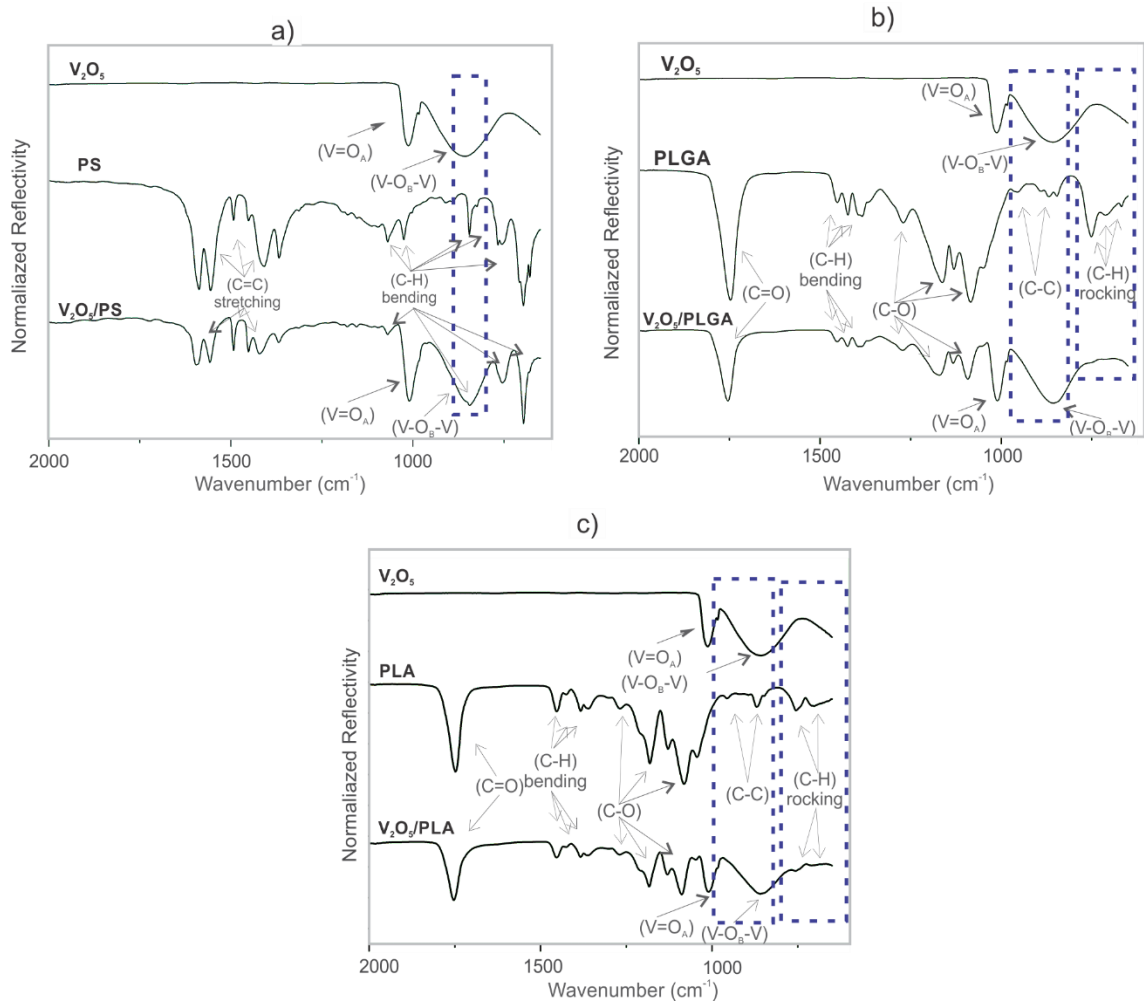


Figure 4.40: ATR-IR spectrum of a) V_2O_5 NWs, PS and V_2O_5 NWs/PS; b) V_2O_5 NWs, PLGA and V_2O_5 NWs/PLGA; c) V_2O_5 NWs, PLA and V_2O_5 NWs/PLA.

The ATR-IR spectrum of the PLGA (Fig. 4.40b) and PLA (Fig. 4.40c) showed the presence of its characteristic bands. The absorption bands at 668 , 712 and 753 cm^{-1}

correspond to the C-H rocking vibrations [144], [145]. The sharp absorption maximum at 1746 cm^{-1} is assigned to the vibration of the carbonyl group (C=O) [144], [145]. The bands at 1271 , 1162 , 1130 , 1084 and 1050 cm^{-1} are assigned to the C-O stretching bond vibration, while the bands at 1452 , 1432 , 1393 , 1384 and 1366 belong to the C-H bending vibrations [144], [145]. Furthermore, the absorption maxima at 956 , 922 , 891 , 868 and 846 cm^{-1} correspond to the C-C stretching vibrations [144], [145]. Not all of the vibrational modes of the V_2O_5 and the PLGA were present in the IR spectrum of the $\text{V}_2\text{O}_5/\text{PLGA}$ (Fig. 4.40b). The peaks corresponding to the C-H rocking vibration are not present (indicated by a dashed rectangle). These vibration bands are present in the IR spectra of $\text{V}_2\text{O}_5\text{NWs/PLA}$, but are strongly reduced in intensity (Fig. 4.40c). It is not clear whether the C-C stretching vibration bands are still present in both, the $\text{V}_2\text{O}_5\text{NWs/PLGA}$ and $\text{V}_2\text{O}_5\text{NWs/PLA}$ composites as they overlap with the strong V-O_B-V band of the V_2O_5 (indicated by a dashed rectangle).

These results indicate the presence of strong intermolecular interactions at the $\text{V}_2\text{O}_5\text{NWs/PLGA}$ interface, as the C-H vibration maxima of the PLGA matrix are overwhelmed. These interphase interactions were also observed within the $\text{V}_2\text{O}_5\text{NWs/PLA}$ composite, but are weaker in intensity than within the $\text{V}_2\text{O}_5\text{NWs/PLGA}$, because C-H rocking vibrations were just partially overwhelmed. In contrast, the IR analysis confirmed the absence of such interactions within the $\text{V}_2\text{O}_5\text{NWs/PS}$ composite.

4.4.2 The dissolution phenomena of the V_2O_5 /polymer composite coatings and their antibacterial properties

In this section, the role of the particle morphology ($\text{V}_2\text{O}_5\text{MPS}$ vs $\text{V}_2\text{O}_5\text{NWs}$) and polymer type (PLGA, Chitosan, PLA, PS) on the elution kinetics of the vanadate from the V_2O_5 /polymer composites to the physiological solution was investigated. The analysis included the observation of composite coatings' surface morphologies after the immersion in the physiological solution and quantification of elution kinetics.

The PLGA matrix was able to reduce the vanadate elution to the PBS, i.e., to control the solubility of the V_2O_5 (Fig. 4.41a). In contrast to the pure powders, the vanadate elution from a composite containing $\text{V}_2\text{O}_5\text{NWs}$ was lower than from one containing the commercial $\text{V}_2\text{O}_5\text{MPs}$ (Fig. 4.41a), i.e., the vanadate level in the medium after $t = 8$ days of exposure to the PBS medium was more than four times lower. The $\text{V}_2\text{O}_5\text{MPs}$ were not observed on the surface of the $\text{V}_2\text{O}_5/\text{PLGA}$ composite after 4 days of exposure to the PBS (Fig. 4.39b). I identified just 200-nm-sized holes at the PLGA surface, because hydrolytic degradation of the PLGA had begun. The formation of up to 1- μm -sized pores due to degradation was also identified on the surface of the V_2O_5 nanowires/PLGA composite (Fig. 4.39d) but the nanowires were still attached to the surface. Just parts of their length were completely covered by the PLGA, i.e. they become exposed to the surrounding PBS medium.

The SEM study of the $\text{V}_2\text{O}_5/\text{PLGA}$ surface indicated that the isotropic $\text{V}_2\text{O}_5\text{MPs}$ eroded from the PLGA surface into the PBS, while the erosion of the $\text{V}_2\text{O}_5\text{NWs}$ was hindered. As a consequence, the elution of the vanadate ions from the $\text{V}_2\text{O}_5\text{NWs/PLGA}$ composite is slower than from the $\text{V}_2\text{O}_5\text{MPs/PLGA}$ composite. Based on the obtained results, I concluded that the 1D nanoparticle morphology of the V_2O_5 allows more efficient control of the vanadate elution compared to the sub-micron particle morphology.

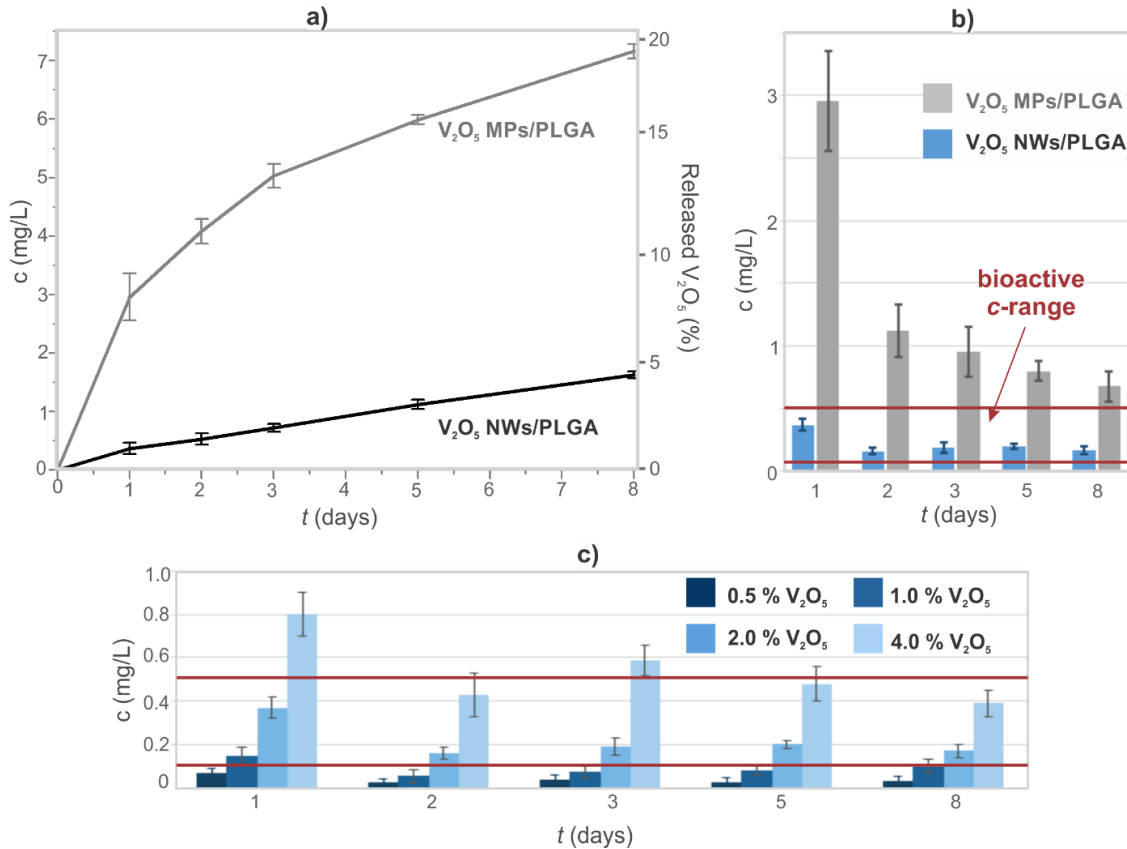


Figure 4.41: a) The cumulative and b) the daily vanadates' elution kinetics upon exposure of the V₂O₅/PLGA composites to the PBS medium; c) b) Daily vanadates' elution from PLGA matrix containing 0.5%, 1%, 2% and 4% of V₂O₅.

The release of vanadates from the V₂O₅ NPs/PLGA does not show any significant initial burst effect and the release of vanadates is constant over time (Fig. 4.41b). The daily release of vanadates was within the bioactive concentration range (within the red marks, Fig. 4.41b). In contrast, initial burst effect of vanadate ions highly above bioactive concentration range was observed from the V₂O₅ MPs/PLGA (Fig. 4.41b).

The daily elution study of the vanadate release from V₂O₅ NWs/PLGA composites confirmed the absence of any initial burst effect (Fig. 4.41c). The maximum allowed amount of embedded V₂O₅ inside the PLGA matrix is 2%, as either higher or reduced loadings resulted in a vanadate release outside of the bioactive concentration range (Fig. 4.41c).

Similar to the V₂O₅ NWs/PLGA, the elution-kinetics of the vanadates from the V₂O₅ NWs/PLA (Fig. 4.42) to the PBS medium showed no initial burst effect, but the final concentration the amount of the released vanadates after 8 days of exposure was almost four times higher compared to the V₂O₅/PLGA composite (Fig. 4.42a). The morphology of the V₂O₅/PLA surface revealed holes in the shape of nanowires after the exposure to the PBS, which implies that the PLA matrix is not efficient in the retention of the nanowires (Fig. 4.39f). In the case of the V₂O₅ NWs/PS composite, almost 55 % of the incorporated V₂O₅ was released after 1 day of exposure to the PBS, while in the next 7 days only 10 % of the material was released, i.e., the strong initial burst effect was observed (Fig. 4.42). The overall concentration of the vanadates was for an order of magnitude above the bioactive range. The morphology of the V₂O₅ NWs/PS surface exhibited holes in the shape

of the nanowires after the exposure to the PBS, proving the burst release of whole nanowire agglomerates (Fig. 4.39h).

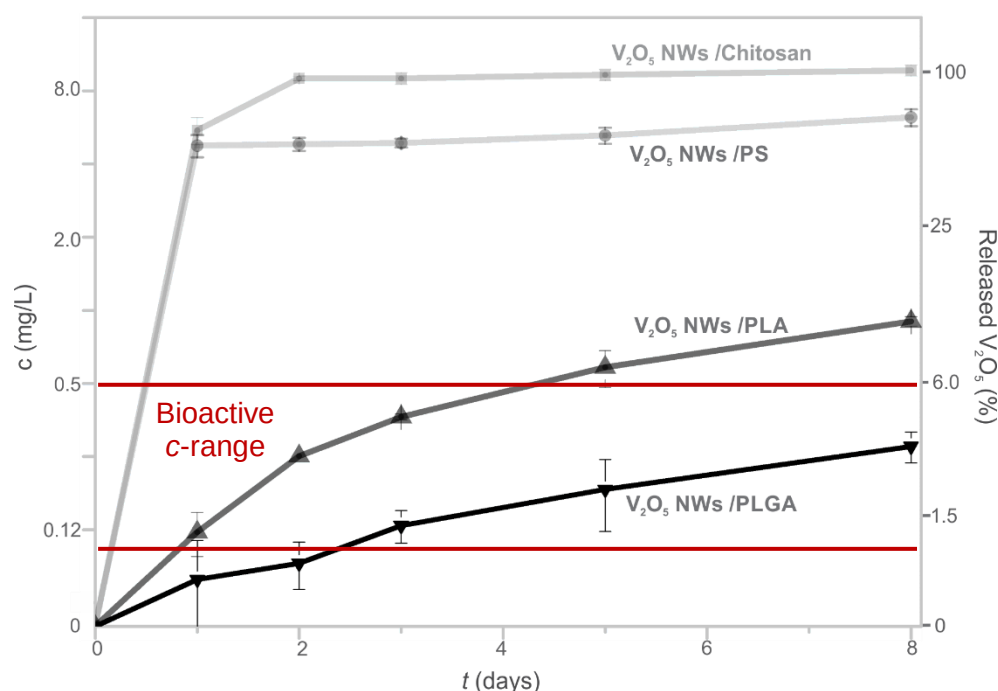


Figure 4.42: a) The cumulative vanadates elution kinetics from different V₂O₅/polymer composites to the PBS medium. The coatings contained 0.5 % of vanadium pentoxide.

The V₂O₅ NWs/chitosan composite exhibited the lowest ability to control the elution kinetics of the vanadates. Namely, more than 90 % of the incorporated nanowires were dissolved from the V₂O₅/chitosan composite after 2 days of exposure to the PBS (Fig. 4.42). In contrast to the other V₂O₅/polymer systems, the coating collapse was observed in the SEM micrograph of the V₂O₅ NWs/chitosan surface, which could be related to the swelling of the matrix and the up-take of water from the medium (Fig. 4.39i).

The study showed that the PLGA, the PLA and the PS differ in their ability to control the dissolution of the nanowires. The polarity of these polymers (based on contact-angle measurements) decreases from PLGA over PLA to PS [122], [151], [152], same as their ability to retain the V₂O₅. Therefore, I anticipated that the polarity of the polymer matrix is related to the strength of the interface interactions at the V₂O₅/polymer interface. In contrast, the chitosan is a highly hydrophilic polymer with a high swelling rate, i.e., the ability to absorb a large amount of water [153]. The obtained results clearly demonstrated that swelling of the matrix has a negative influence on the control of vanadate elution from the V₂O₅/chitosan composite.

4.4.3 The antibacterial properties of the V₂O₅/polymer composite coatings and their hemocompatibility

The ability of the V₂O₅ NWs/PLGA composite coatings containing 2 % of V₂O₅ to prevent bacteria colonization and biofilm formation at the surface was evaluated. To evaluate an extent of bacterial colonization of the coatings surface, the solution above the evaluated coatings was smeared on agar plates after vigorous stirring. After an overnight incubation no colony growth was observed at the agar plates. This indicated the complete colonization

of the *S. epidermidis* from the initial suspension to the surface of the coatings. However, a difference in the number of adsorbed bacteria was observed. The CV assay showed that the amount of colonized bacteria on the surface of the V_2O_5 /PLGA composite coatings was more than three times lower than at the surface of the PLGA coating. (Fig. 4.43a). The *live/dead* assay revealed that the majority of the colonized bacteria at the surface of the V_2O_5 /PLGA composite were dead because the red signal was much stronger than the green signal. In contrast, a significant green signal is observed at the surface of the PLGA, which proved the presence of live bacteria at the surface of the PLGA (Fig. 4.43b).

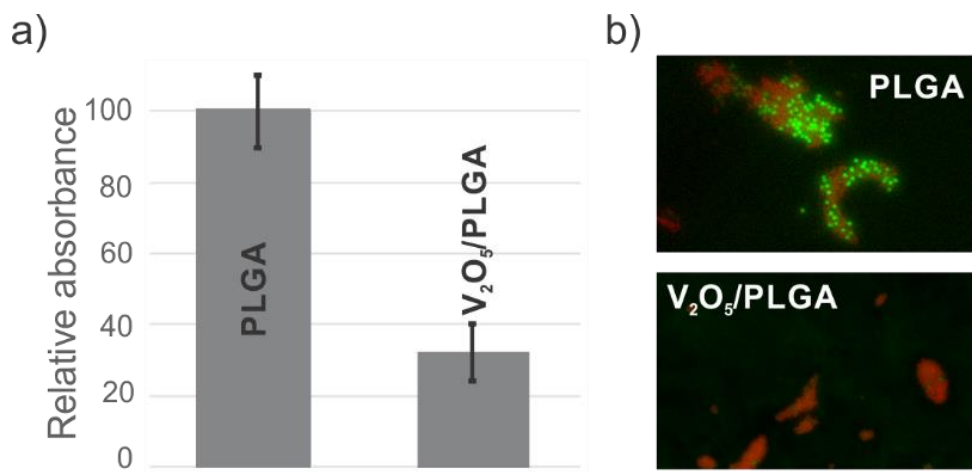


Figure 4.43: a) The relative absorbance at 590 nm of extracted crystal violet solutions and b) *BacLight live/dead* imaging of attached *S. epidermidis* at the surface of the coatings. Composites contained 2% of vanadium pentoxide.

These results confirmed the ability of the V_2O_5 to prevent bacteria colonization of the PLGA surface, while at the same time vanadate release within bioactive concentration limits was confirmed.

Because RBCs would be the first cells interacting with the V_2O_5 NWs/PLGA composite coating, I evaluated the structural and morphological changes of RBCs upon exposure to the coating. After a 24 h long incubation of the prepared coatings with the blood, the amount of released haemoglobin was measured. Using the previously prepared calibration curve (Fig. 4.38a), the percentage of hemolysed RBCs was determined. When RBCs were exposed to V_2O_5 NWs, 65 % of them were hemolysed. In contrast, exposure of the PLGA coating, as well as to V_2O_5 NWs/PLGA coatings with either 2 % or 1 % of V_2O_5 , to the blood solution did not cause hemolysis of RBCs (Fig. 4.44a).

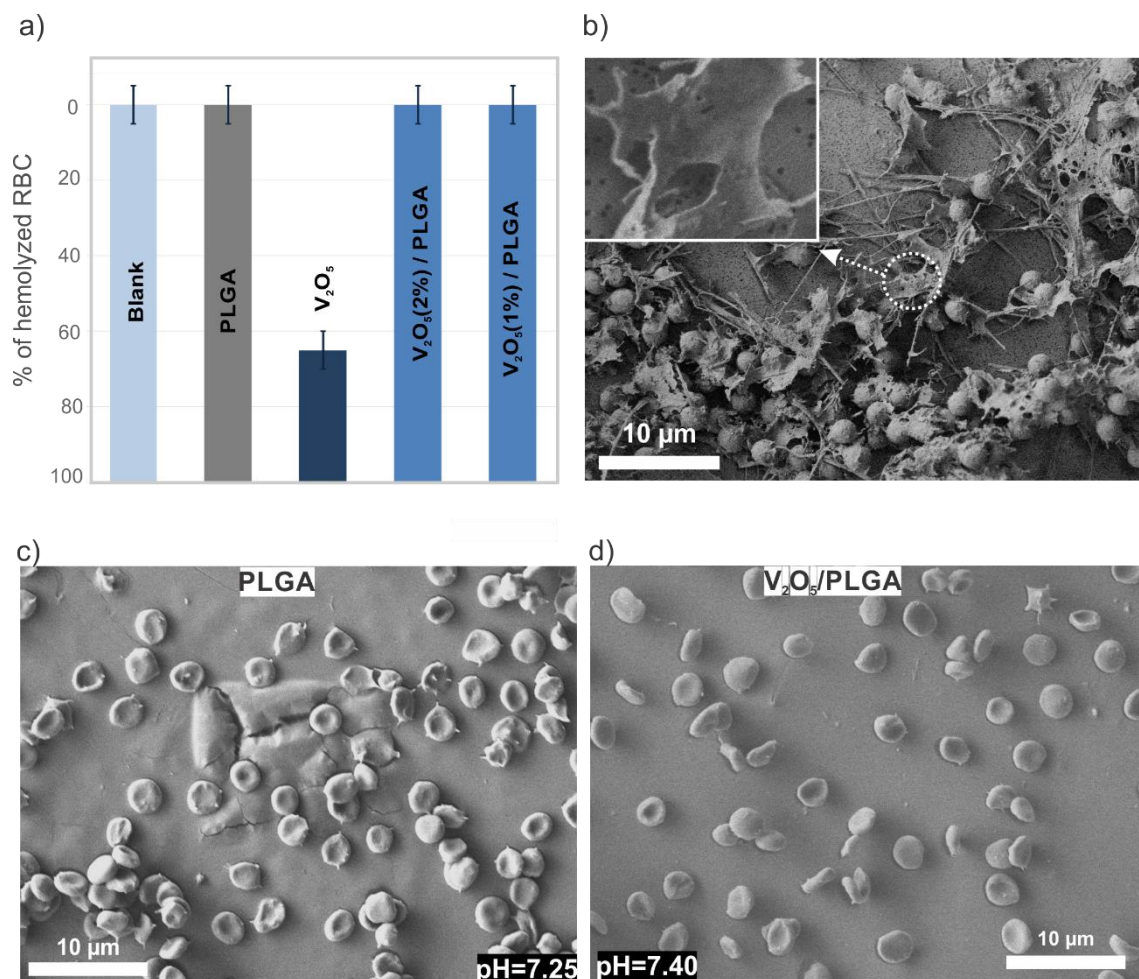


Figure 4.44: a) The % of hemolysed RBCs after a 24 h long exposure to the various composite coatings. The morphology of the RBCs at the surface of either c) PLGA or d) $V_2O_5(2\%) / PLGA$ coatings observed by the SEM microscopy. The pH of the RBCs suspension after the exposure is provided on the SEM images.

To evaluate the impact of the RBCs/material interactions on the cell morphology, they were observed by the SEM. When the cells were exposed to the V_2O_5 nanowires, we observed severe membrane rupture (Fig. 4.44b), which is in accordance with hemolysis measurements. Introduction of 2 % of V_2O_5 NWs within the PLGA matrix resulted in both, annihilation of RBCs hemolysis and the reduction of the number of RBCs with crenated shapes attached at the coating surface when compared to the RBCs at the PLGA surface (Fig. 4.44c, d). At the same time, the supernatant pH after the V_2O_5 NWs /PLGA coating was incubated in blood suspension in PBS was 7.4, which is the same as in the PBS, i.e. introduction of V_2O_5 NWs within the PLGA matrix, similar to the introduction of the MgO, reduced PLGA's negative effect to the RBCs. Obtained results demonstrated that the composite coatings containing 2 % or less of V_2O_5 NWs neither cause mechanical damage of the RBCs nor release of the haemoglobin.

Chapter 5

Discussion

5.1 Structural Characteristics of Nano-Textured Magnesium Oxide Microrods and Their Interactions with Bacteria and Surrounding Media

The variation in the calcination temperatures allowed a deep insight into the formation of the nano-texturing on the surface of the MgO microrods, as an effective tool for improving their contact-based antimicrobial activity. During the thermal decomposition, the precursor particle shape was preserved. At lower temperatures (below 700°C), the formed MgO crystallites were small and as a result the surface of the prepared MgO microrods was not nano-textured (Fig. 5.1). An increase in thermal decomposition temperature (700°C or higher) resulted in larger sizes of formed MgO crystallites, causing the nano-texturing of the microrods (Fig. 5.1). The obtained results are in accordance with the previous studies as the particle shape (i.e. microrods) was preserved during the thermal processing, while the surface is modified during the high-temperature processing [43], [46], [61], [154].



Figure 5. 1: The evolution of nano-texturing at the surface of the MgO microrods.

The variation of (i) **the atmosphere** (air or nitrogen) during the thermal processing of precursor microrods or (ii) **the annealing** in air of the already prepared powders allowed manipulation of the MgO surface at the level of atomic defects, which was a useful tool for studying molecular basics of MgO antibacterial activity. I observed no change in the morphology of obtained powders due to differences in thermal processing. AN MgO NPs exhibited same morphology as MgO NPs while MgO 900 N2 exhibited the same morphology

as MgO 900 microrods, which was in accordance with the literature reports, where both, the annealing [57] and variation of processing atmosphere [46], did not affect the particle morphology.

In these reports, it has been reported that surface defect peculiarities were strongly affected by annealing or with the variation of the thermal decomposition atmosphere. For example, the annealing of MgO nanoparticles caused decomposition of surface magnesium hydroxide as well as reduction of native defect abundance [57]. Our results confirmed this observation, as BET area of the studied MgO NPs was reduced upon annealing (from 35 to 32 m²/g). Their annealing, as determined during analysis of AN MgO NPs by means of IR (Fig. 4.7b) and TGA (Fig. 4.8), caused an absence of high-energy bonded isolated OH groups at the surface. These groups preferentially bond at kinks and step di-vacancies (3C sites surface defect sites) [49], [74]. Thus, the annealing of the MgO NPs caused an annihilation of kinks and step divacancies, which could explain the identified change in type of 3C oxygen atoms (red shift in UV absorption maximum, Fig. 4.5b) as well as an increase in the abundance of 4C oxygen atoms. Such observations strongly point towards MgO surface reconstruction upon annealing.

The effect of the thermal decomposition atmosphere on the atom arrangement of MgO microrods on the surface has been reported [46]. The nature of basic sites on the MgO surface changed when particles were processed in nitrogen instead of air. The processing temperatures were lower than in our study (500 and 700°C [46] vs 900°C), which resulted in differences among MgO IR spectra. Powders processed at lower temperatures (both 500 and 700°C) still possessed a sharp vibration band corresponding to isolated OH groups [46], while processing at higher temperatures caused diminishing of isolated OH groups at the surface of either MgO 900 or MgO 900 N₂ (Fig. 4.7b).

The defect equilibria thermodynamics could be useful to predict impact of a processing atmosphere on the surface peculiarities of MgO. The amount of created oxygen vacancies is inversely proportional to the oxygen partial pressure[155] according to Eq. (5.1).



Therefore, the concentration of oxygen vacancies would be several orders of magnitude higher when MgO was processed in nitrogen instead of in air, leading to oxygen deficient material, which explains the abundance of oxygen vacancies and oxygen vacancy dimmers in the MgO 900 N₂ powder (Fig. 4.5 c, d) as well as the lower concentration of LC oxygen atoms at the surface of MgO 900 N₂ than at the surface of MgO 900 (Fig. 4.5b). As a result, MgO 900 N₂ exhibited a higher BET area than MgO 900 (14 vs 6.6 m²/g).

Although MgO microrods are less agglomerated than the NPs, it was crucial to modify the surface of MgO microrods to achieve a comparable antimicrobial activity. In the literature studies regarding optimization antibacterial activity of the MgO nanoparticles was a maximum for particles with the highest specific surface area [59] or for the particles with the highest concentration of oxygen vacancies [156]. In these studies the comparisons among various MgO powders were made for the nanoparticles with a different average particle size. Thus, I firstly investigated the effect of nano-texturing of MgO microrods (keeping the defect peculiarities constant) on the antibacterial activity. Secondly, the particle morphology was kept the same to allow determination of the influence of surface defects nature and abundance at the antibacterial potential of MgO.

The nano-texturing improved the antimicrobial activity of the MgO microrods and led to the optimal nanostructures, which were obtained after processing at 900°C (BET area = 6.6 m²/g, Fig. 4.3e). Such an observation does not completely match the trends of the specific surface of nano-textured microrods, as the highest BET value was observed for the

MgO 700 (BET area = 7.5 m²/g, Fig. 3c). The disc-diffusion method coupled with an evaluation of the effects of Mg²⁺ and pH on the *E. coli* growth kinetics proved that, similar to the MgO slurry particles [56], the nano-textured MgO microrods exhibit a contact-based mechanism of antibacterial activity, i.e. the effectiveness of the MgO/bacteria contact is governing the antibacterial activity of nano-textured MgO microrods. The differences in antibacterial activity between MgO 700 and MgO 900 can only be attributed to their surface characteristics. As the UV diffuse reflectance spectra of the MgO 700 and MgO 900 coincided, the difference in the specific surface area (7.5 vs 6.6 m²/g, respectively) depended only on the nano-texturing of MgO microrods. The MgO 700 microrods exhibited the largest specific surface area, but the surface features were small to allow an optimal contact with the *E. coli* (Fig. 5.2). On the other hand, the MgO 1000 microrods exhibited surface features that were too large (Fig. 5.2). The optimal contact, and thus, the optimal activity against *E. coli*, was achieved for the nano-textured MgO 900 microrods, where the crystallite size was 41 nm (Fig. 5.2). As-prepared particles were more effective against *E. coli* than the commercial MgO NPs, which was proved by both, MDT method (Fig. 4.9a,c) and *BacLight live/dead* (Fig. 4.10a), methods. I concluded that to achieve optimal antibacterial activity, it is necessary to design MgO surface in such a manner to obtain optimal contact with bacteria.

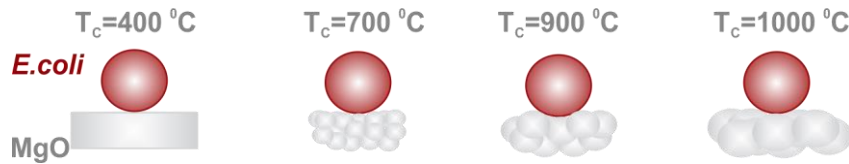


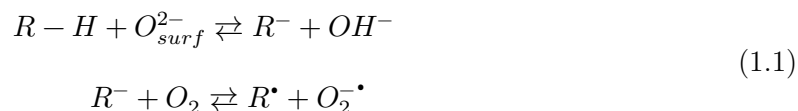
Figure 5.2: The nano-texturing of the MgO microrods and contact with the bacteria.

The surface defects nature and abundance also played a significant role. MgO 900 N2 particles were more active than the MgO 900 microrods. The only difference among them was the high concentration of the oxygen vacancies at the surface of MgO 900 N2 microrods, i.e. MgO 900 N2 microrods were more oxygen deficient than MgO 900. The weaker antibacterial activity of the MgO NPs and AN MgO NPs (high concentration of low-coordinated oxygen at the surface) when compared to MgO 900 microrods further provides proofs that oxygen deficiency of MgO surface positively influences the antibacterial potential of the MgO. The difference in antibacterial activity of MgO NPs and AN MgO NPs was significant. The most pronounced difference among the studied powders was the high presence of kinks and di-step vacancies at the surface of MgO NPs compared to low presence of such defects at the surface of AN MgO NPs. As the AN MgO NPs were more antibacterially potent than MgO NPs, I provided evidence that annihilation of kinks and step divacancies by annealing improves antibacterial activity. Thus, apart from the optimal nano-texturing, activity of MgO microrods can be improved by creation of the oxygen-deficient surface (by processing in the nitrogen) and securing the annihilation of kinks and step di-vacancies (high temperature processing and/or annealing).

The impact of the various surface defects peculiarities on the ROS generation potential of the studied MgO powders provided valuable information about the underlying mechanism of MgO antibacterial activity. In literature, ROS generation was attributed to either reduction of oxygen at oxygen vacancy [56], [62] or at specific defect sites [57], [62]. However, the source of energy is required (e.g., UV irradiation) to trigger the reduction of the molecular oxygen at the oxygen vacancy [157] and I demonstrated by means of the in-situ fluorescence DHR assay method that MgO 900 N2 did not produce any oxidative species (Fig. 4.11a, b), irrespective of the high concentration of surface oxygen vacancies. As the MgO NPs were the only species to exhibit ROS generation upon incubation in

aqueous environment, I concluded that high-energy, low-coordinates surface sites (e.g., step di-vacancies and kinks) are responsible for the inherent ROS generation of MgO powders during incubation in aqueous media. The same results were obtained when the tendency of MgO NPs and MgO 900 powders to produce ROS species was evaluated by means of EPR spectroscopy. In contrast to the positive control, the EPR signal of the produced DMPO-OH radical was increasing with the incubation time of the MgO NPs. I concluded that the ROS generation was related to the MgO hydrolysis as both processes were progressive during the incubation in a physiological solution. Such result indicates the differences in the mechanism of hydrolysis between the MgO NPs (that contain step di-vacancies and kinks) and other studied MgO powders (that do not contain step di-vacancies and kinks). As all of the powders exhibited a strong bactericidal activity, the obtained results were in accordance with the recently reported non-ROS mechanism of antibacterial activity for the MgO nanoparticles [57].

When the *E. coli* were added to the solution, the significant ROS generation upon MgO/bacteria contact for all of the tested powders was measured using the fluorescent method (Fig. 4.11c, d). Similarly, after 30 minutes of incubation, the MgO 900 particles with *E. coli* showed traces of DMPO-OH formation, confirming ROS generation (Fig. 4.12c). Thus, the results obtained using both methods indicated the generation of ROS species as a side product of the MgO/bacteria interactions. Taking into account the ability of the *E. coli* to metabolise the ROS into non-oxidative products (Fig. 4.11c, d) and the unaltered antibacterial activity of the MgO NPs when the ascorbic acid (ROS inhibitor) was introduced to the MgO/bacteria suspension (Fig. 4.13), I concluded that the ROS species were formed as a side product of the MgO/bacteria interactions, which could explain both the expression of stress-related genes upon MgO/bacteria interactions [63] as well as the presence of the non-ROS mechanism for the MgO antibacterial activity [57]. The generation of ROS species as side products of MgO/bacteria interactions could be explained by the formation of superoxide ions at the MgO surface through the agency of previously absorbed organic molecules, according to Eq.(1.1) [64], [157].



The presorbed molecules could be benzene, toluene, ammonia, ethylene, acetylene etc. [64], [157] where the basic nature of MgO causes ionic cleavage of C-H and N-H bonds of absorbed molecules. Thus, I believe that chemical acid/base reaction between MgO surface atoms and macromolecules in bacteria wall is a molecular mechanism of antibacterial action of MgO apart from the previously proposed physical interactions of MgO with the bacterial wall molecules (e.g. lipopolysaccharides) [57]. Nevertheless, further studies dedicated to a more precise evaluation of the acid/base reaction mechanism between the MgO surface and the components in the bacterial wall are required.

The comprehensive study on the dynamics of hydrolysis and dissolution of the studied MgO powders provided valuable information about the impact of various surface defects on the antibacterial properties of the MgO. The phase transformation of the MgO to Mg(OH) in physiological solution has been reported after 24 h of exposure [82]. Regarding the underlying mechanism, it was reported that water hydrolysis of MgO was propagating via the erosion at corner sites [78], [158] i.e. via the O_{3C} - Mg_{4C} sites. The simulation of the hydrolysis energies at various MgO surface defect sites by means of Periodic Density Functional (PDF) theory showed that acid/base nature of MgO is increased by lowering the coordination of the surface atoms. The coordination of the oxygen atoms (L') at the surface tends to influence the basicity of the surface Mg_{LC} - O_{LC} pair stronger than the

coordination of magnesium atom(L) [49]. In our study, I obtained a completely transformed hydroxide phase after 16 h for all of the evaluated MgO powders (Fig. 4.14). I related the significant differences in the kinetics of oxide-to-hydroxide transformation (Fig. 4.15a) among the studied MgO powders to surface defects peculiarities. The MgO NPs, which transformed faster than the AN MgO NPs, possess a higher concentration of oxygen terminated kinks and di-step vacancies in comparison with AN MgO NPs. These surface defects are considered as strong acid-base sites [49] and I believe that they act as a nucleation spots for hydroxide phase transformation. As a result, the number of nucleation spots was higher in the case of MgO NPs than for the other three powders, which caused smaller crystallite size of the formed hydroxide phase after hydration of MgO NPs compared to the other three powders (Fig. 4.15b). The difference in oxide-to-hydroxide transformation kinetics between the MgO 900 N2 and MgO 900 microrods provided another proof about the importance of surface oxygen in the hydration of MgO. MgO 900 N2 microrods exhibited a slower hydration rate due to the high abundance of surface defects.

As hydration preferentially proceeds via the O_{3C} - Mg_{4C} sites, I visualized the impact of oxygen deficiency on the MgO hydration via the following hypothetical experiment. One O_{3C} site was located at the ideal MgO cube edge (Fig.5.3a). The number of these sites was increased to two (Fig. 5.3b, dissolution of two MgO molecules) and four (Fig. 5.3c, dissolution of eight MgO molecules). However, oxygen deficient MgO cube did not exhibit an O_{3C} site at its surface (Fig. 5.3d). After the removal of two water molecules, the first O_{3C} was exposed to the environment (Fig. 5.3e). Due to oxygen deficiency, the number of O_{3C} was just two (Fig. 5.3f), when compared to four in the case of a non-oxygen deficient cube (Fig. 5.3c).

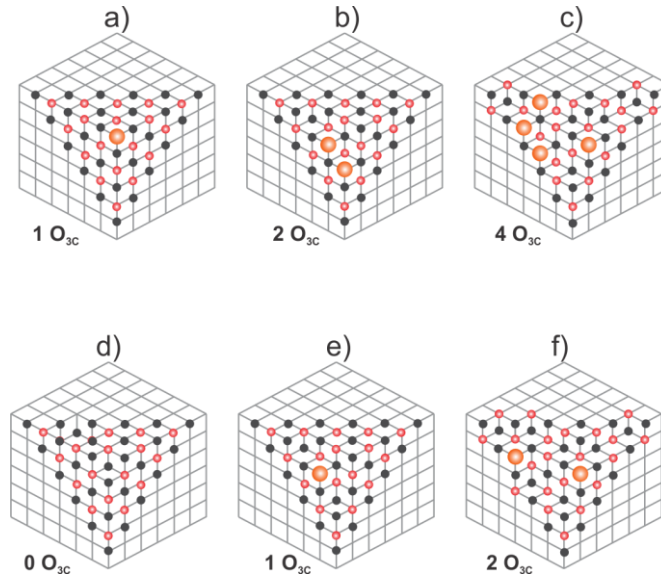


Figure 5. 3: The surface atom arrangement of ideal MgO cube a) before exposure to aqueous environment, b) after dissolution of two MgO molecules and c) after dissolution of eight MgO molecules. The surface atom arrangement of oxygen deficient MgO cube d) before exposure to aqueous environment, e) after dissolution of two MgO molecules and f) after dissolution of eight MgO molecules.

These findings proved the necessity of (i) **annihilation** of oxygen terminated kinks and step-di vacancies and, (ii) **reduction** of oxygen concentration on the surface of MgO in order to improve its resistivity to hydration. These are the same requirements as for the

optimal antibacterial activity of MgO powders. As a consequence, I concluded that there is a relation between antibacterial activity and hydration behaviour of MgO particles. Antibacterial characteristics of MgO particles are usually measured in an aqueous environment. Because of that, hydration is taking place as MgO powder is exposed to action of water molecules. When it is faster, more of the MgO is dissolved and transformed to hydroxide, causing a reduction in an amount of the MgO solid phase to interact with bacteria. Although the hydration kinetics is site specific (it is not occurring at 5C sites) [45], this was not the case with the acid/base molecular mechanism of the MgO antibacterial activity (Eq. 5.2) because the interactions of MgO surface with N-H and C-H molecules are not site specific [160].

The hydration process of the MgO powders is different in different media. The solubility product of the MgPO_4 is lower than the solubility product of the $\text{Mg}(\text{OH})_2$, which implies preferential precipitation of the phosphate phase compared to the hydroxide phase. As a consequence, 1D microparticles of the phosphate phase were firstly precipitated (Fig. 4.17, Fig. 4.18, Fig. 4.19), when MgO NPs and MgO 900 microrods are exposed to the PBS. The process was proceeded by a dissolution of MgO and release of the Mg ions from the particle surface. The progression of the phosphate phase precipitation was enabled by the dissolution of MgO particles which resulted in a release of Mg ions. The precipitated Mg-phosphate phase became amorphous upon the prolonged exposure to the surrounding aqueous media. The reason was unstable nature of Na and K within the initially precipitated Mg-phosphate phase ($t = 2\text{h}$) and their release to the medium ($t = 24\text{h}$).

Once all of the available phosphate ions were consumed during the Mg-phosphate phase precipitation, further dissolution of MgO particle surface led to increase in Mg^{2+} and OH^- ions concentration up to the solubility product of the $\text{Mg}(\text{OH})_2$. At that point ($t = 24\text{h}$), topotactic oxide-to-hydroxide phase transformation started at the surface of the remaining MgO particles. Because a large number of MgO molecules was removed from the particles' surface up to this point, the surface is highly defected resulting in a large number of nucleation sites and a smaller size of the formed hydroxide phase (9-10 nm) when compared to the transformation in the physiological solution (15-35 nm).

The presence of proteins in the MgO suspension causes formation of protein corona around the MgO particles. Literature reports demonstrated that as-formed corona was successful in the prevention of the MgO hydration within 24 h of exposure to cell-growth media and was unsuccessful upon MgO particles exposure to the LB growth media [82]. I monitored the structural transformation's dynamics during the long-term exposure (96 h) of the MgO in cell growth media. The observed initiation of oxide-to-hydroxide transformation after 96 h of exposure clearly proved that bonding of proteins at the surface of MgO does not provide ultimate protection against the oxide-to-hydroxide transformation but rather slow down the process. The presence of phosphate ions in the solution and precipitation of phosphate phase (exposure to MH medium) proves that, even though the MgO particles were covered with protein corona, the dissolution of MgO from the particle surface towards the medium still proceeds. However, due to the unavailability of the surface, the topotactic growth of hydroxide phase is not possible and as a result after 96 h of exposure to MH medium, the MgO phase was completely amorphized.

5.2 Structural Characteristics of Nanostructured Vanadium Pentoxide and Their Interactions with Bacteria and Surrounding Media

The impact of the structural and morphological characteristics of the hydrothermally processed 1D nanostructured vanadium pentoxide powders on their biomimetic antibacterial activity and dissolution properties provided valuable information about their applicability. Even though materials were processed at the same temperature, all of the synthesized V_2O_5 powders exhibited a longer aspect ratio than powders described in the literature and prepared at the same conditions. The reason was the difference in a reactor assembly, which pointed out toward a conclusion that kinetic effects (e.g. heat transfer rate) during the synthesis of the nanostructured vanadium pentoxide powders have an important role on the final characteristics of the synthesized powders.

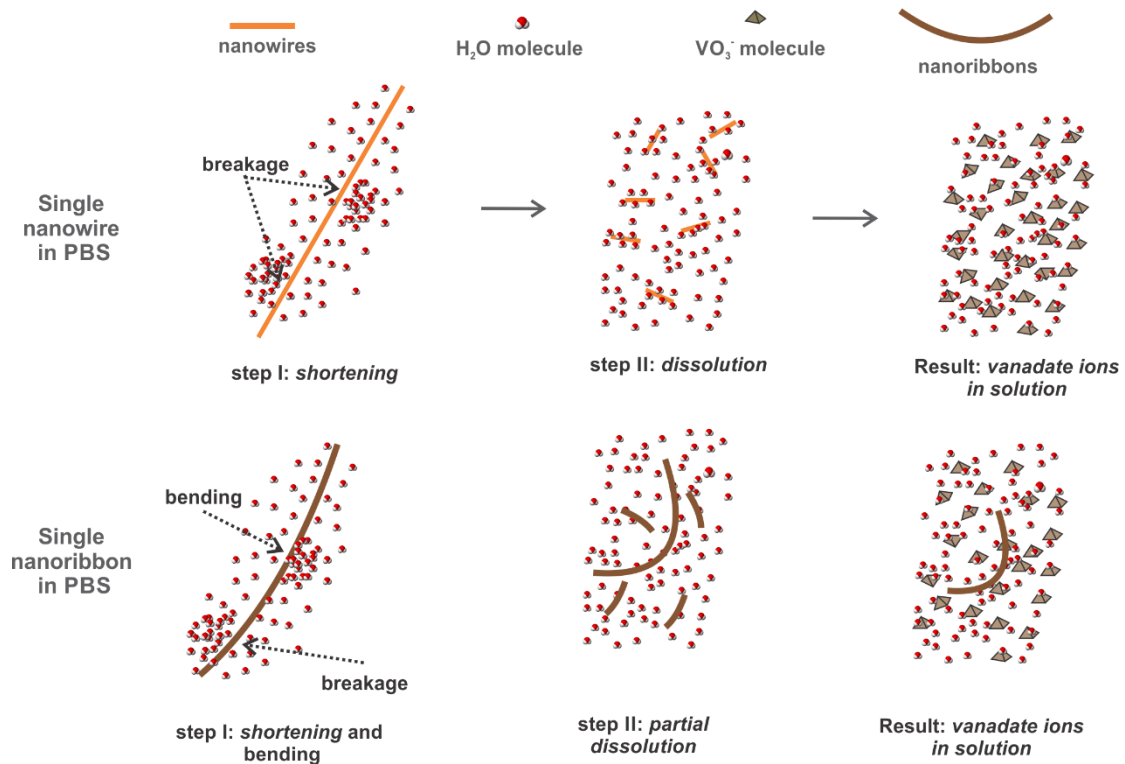


Figure 5.4: Mechanism of dissolution of vanadium pentoxide nanowires and nanoribbons in the PBS solution.

Based on the obtained findings related to dissolution kinetics of 1D nanostructured V_2O_5 particles (Fig. 4.28) and morphological changes upon the exposure of V_2O_5 NWs to aqueous solution (Fig. 4.29), I proposed a dissolution mechanism of the nanostructured V_2O_5 particles (illustrated in Fig. 5.4). When exposed to the PBS medium, a single vanadium pentoxide nanowire interacts with the surrounding water molecules. Due to water flows at a micro level, induction of local sheer stress on the nanowire is occurring. Because of the large aspect ratio, the nanowire is broken at several positions along its length, i.e. radial wire length reduction occurs. During the second step these remainings completely dissociate to vanadate ions, as the length of the nanowires did not significantly change from the 1st to the 8th day of exposure to an aqueous media (Fig. 4.29). The V_2O_5 NWs exhibited more pronounced dissolution rates than $K_{0.33}V_2O_5$ and $V_2O_5 \cdot xH_2O$ (Fig.

4.28). The SEM image of particle morphology indicates the tendency of nanoribbons (both, $K_{0.33}V_2O_5$ and $V_2O_5 \cdot xH_2O$) to bend (Fig. 4.25c, d). Because of that, these nanoribbons were more resistant to the water micro flows in the solution and their radial breakage was reduced compared to vanadium pentoxide nanowires (Fig. 5.4).

Regarding the antibacterial potential of V_2O_5 , I related it completely to its ability to catalyse the transformation of hydrogen peroxide to hypochlorite ions via a two-stage mechanism. At first, the V_2O_5 produces a small amount of hydrogen peroxide, as previously suggested [159]. I have confirmed that this amount is not sufficient to affect the bacteria, as their viability was not reduced (Fig. 4.30a). In the presence of both, chloride ions and V_2O_5 , the formed small amount of hydrogen peroxide is transformed to hypochlorite ions, which effectively eliminate the bacteria (Fig. 4.30a, b). However, the external addition of hydrogen peroxide (10 μM) to the solution reduced the antibacterial potential of the V_2O_5 . That was a consequence of the accelerated dissolution of V_2O_5 in the presence of added external H_2O_2 , resulting in the reduction of available V_2O_5 nanowires' surface for catalysis as compared to the suspension of V_2O_5 nanowires in the phosphate buffer containing just chloride ions. Although the ROS production is common for metal oxides [160], I proved that V_2O_5 has a unique ability to transform it to more potent hypochlorite ions.

The better resistance of nanoribbons to dissolution in comparison with nanowires, the biomimetic myeloperoxidase-based antibacterial activity of the nanowires was stronger. In general, the high temperature annealing of $V_2O_5 \cdot xH_2O$ particles led to a pure orthorhombic vanadium pentoxide phase which is more catalytically active. In this study, I proved that the same is true for the vanadium pentoxide nanowires prepared at low temperatures in the hydrothermal reactor. Based on these results, the improvement in the dissolution resistance rate of the nanoribbons ($K_{0.33}V_2O_5$ and $V_2O_5 \cdot xH_2O$) was not sufficient to prevail their weaker catalytic activity in comparison with V_2O_5 NWs.

5.3 The Properties of the Magnesium-Oxide/Polymer Composite Coatings in *in-vitro* Conditions and Interaction with the Biological Systems

The incorporation of the MgO within polymer based coatings was scarcely studied. There is a lack of comprehensive studies designated to determine the structure-stability-activity correlation. Therefore, the determination of the impact of the (i) **particle morphology** and (ii) **polymer physicochemical characteristics** on the solubility control of the MgO within polymer matrix provided essential guidelines regarding the design of the MgO/polymer matrix composites.

The improvement of the physical properties of the MgO-NPs/polymer systems was achieved by the addition of the third component, dedicated to enhance more regular dispersion of the nanoparticles within the polymer matrix [116], [117]. In this study, a more sustained release and prevention of initial burst effect of magnesium from the MgO/polymer coating was achieved, simply by applying the 1D microrods instead of the highly agglomerated MgO NPs. The first reason is better processing characteristics of the 1D microrods due to their less expressed particle agglomeration compared to the agglomerates of MgO NPs. The other factor is impact of the matrix degradation on the erosion of the MgO particles from the matrix surface towards the surrounding media. I observed a pronounced degradation of the PLGA matrix in the vicinity of the inserted MgO particles. The holes on the MgO/PLGA composites' surface were rounded in shape (Fig. 4.33a, b). Because of that, whole agglomerates of the MgO NPs were released to the surrounding media. In contrast, due to their larger aspect ratio, MgO 900 microrods were

just partially exposed to the media and withheld within the matrix. Thus, retention of 1D MgO particles was more successful than the retention of isotropic agglomerated MgO nanoparticles within the PLGA matrix.

The improved processability of MgO 900 microrods and their more effective retention at the surface of PLGA matrix when compared to the agglomerated MgO NPs also exhibited the beneficial effect on the antibacterial characteristics of MgO/PLGA polymer coatings. As-prepared MgO900/PLGA coatings eliminated 12% more *E. coli* in suspension than the MgO NPs/PLGA, which confirmed correlation between better MgO particle processability and antibacterial activity. After 3-days of exposure in the physiological solution, the efficiency of MgO 900/PLGA coatings in the elimination of bacteria was more than double of the MgO NPs/PLGA coating's efficiency. Thus, retention of MgO/PLGA antibacterial activity depends on the retention of the particles within the polymer matrix and sustained release of magnesium to the surrounding media.

The retention of the MgO 900 microrods at the surface of the PLGA matrix is baked by the interphase MgO/polymer interactions. When these interface interactions were not sufficiently strong, MgO 900 microrods were assembled in aggregates at the PCL surface (Fig. 4.31d). The IR spectra analysis (Fig. 4.32) suggests interactions between the C-H group of the polyester and MgO particles. The inductive effect of the ester group causes generation of the partial positive charge at C-H group (Fig. 5.5). These groups then interact via dipole-dipole interactions with the surface of the MgO or more precisely with the partially negative oxygen surface atoms. The inductive effect is transferring the charge along the whole monomeric units. The monomeric unit of glycolic acid unit possesses five, the unit of lactide acid possess eight, while the caprolactone unit possesses sixteen of both, C-C and C-H bonds. As a consequence, the induced partial positive charge is the highest in the case of the PLGA and the lowest in the case of the PCL, which governs the strength of dipole-dipole interphase interaction to decrease in the following order MgO900/PLGA > MgO 900/PLA > MgO 900/PCL. Apart from the described influence on the surface morphology of the MgO 900/polymer composites after exposure to the physiological solution and IR spectra, the strength of these interactions plays an important role during the preparation procedure for the composites. In the case of PLGA and PLA, dipole-dipole interphase interactions help stabilize the nanowires in the polymer solution by preventing extensive re-agglomeration (Fig. 4. 31b, c). When they are weaker, the microrods re-agglomerate like observed at the surface of the MgO 900/PLA (Fig.4. 31d).

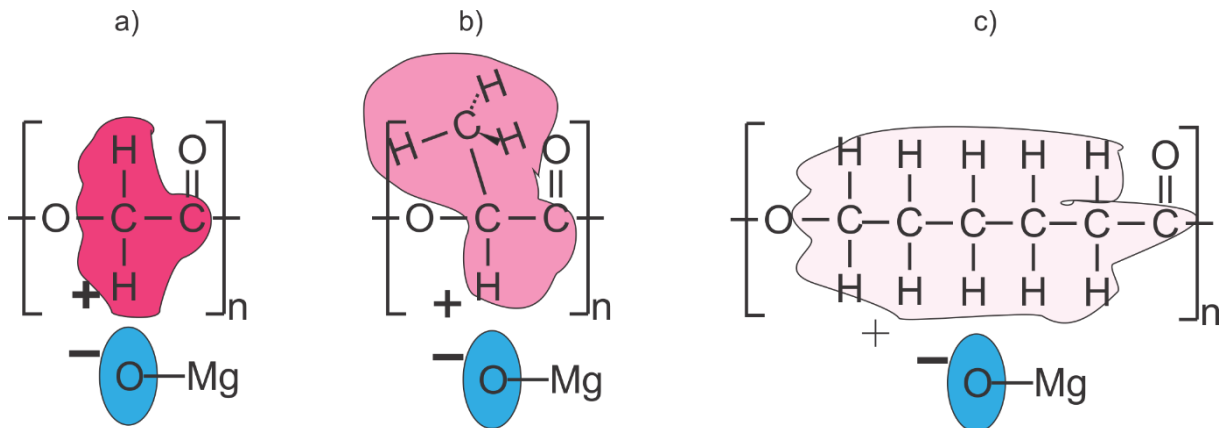


Figure 5.5: Inductive effect of C=O and C-O polyester bonds on C-H bonds and dipole-dipole interactions a) lactide unit, b) glycolide unit and c) caprolactone monomeric unit with MgO particles.

S. epidermidis causes 32 % of all the implant-related bacterial infections (a leading threat together with *S. aureus*) [27], which makes it a representative bacterial species for evaluating the antimicrobial potential of MgO to protect the PLGA surface. The bacteria concentration used in the evaluation of the MgO 900/PLGA antibacterial potential was two orders of magnitude higher than proposed by ISO 22196:2007(E) because the use of lower concentrations led to experiments with large standard deviations. Resazurin was commonly used within standard Alamar Blue assay for determination of the metabolic activity of cultured cell line systems. The measurement involves treatment of the attached cells at the bottom of an individual well with a resazurin solution. When cells are metabolically active, they reduce the resazurin to fluorescent resorufin and the intensity of the fluorescent emission is proportional to the metabolic activity of the cells [131]. I exploited the same principle for the bacteria attached at the surface of the prepared coatings. The obtained method was sensitive and clearly demonstrated strong metabolic activity of the bacteria attached at the surface of the PLGA. In contrast, the bacteria attached at the surface of MgO 900/PLGA were not metabolically active. In addition, the viability of the attached *S. epidermidis* at the surface of the V₂O₅/PLGA was completely reduced when compared to the PLGA surface. Thus, the presence of MgO within the PLGA matrix reduced both the metabolic activity and viability of the attached bacteria. The obtained results confirmed ultimate efficiency of MgO to protect PLGA surface against *S. epidermidis* colonization.

The implanted biomaterial is firstly in contact with blood, i.e. or more precisely, blood related proteins and red blood cells [4]. During the process, the provisional protein matrix is established and it mediates the contact with other recruited cell systems. Therefore, the impact of the MgO/PLGA composite on the RBCs in the presence of blood related proteins was evaluated as an initial method for determination of MgO/PLGA cytotoxic potential. The main difference in the activity of the MgO against bacteria and cells is the impact of medium pH. Opposite to the bacteria, a pH rise (due to MgO dissolution) causes the increased reduction of the cell viability [72]. Because of that, the HEPES buffer was added to the physiological solution. The high amount of MgO within the coatings (>25 %) proved to be too high, as it induces rise of the pH of the medium and hemolysis of the RBCs. When reduced to below 10%, it showed no pH rise and consequently the absence of adverse effects on RBCs neither in suspension nor at the surface of the MgO/PLGA. These results confirmed hemocompatibility of the MgO/PLGA coatings, as the attached red blood cells exhibited no change in morphology.

The coupling antibacterial properties of MgO and bioactive properties of magnesium ions were encouraged as an advanced approach for improvement of the healing processes in the bone tissue engineering [72]. I proved that as-prepared MgO/PLGA coatings are a promising formulation for further testing in *in-vitro* conditions involving osteoblast and fibroblast cell lines.

5.4 The Properties of the Vanadium-pentoxide/Polymer Composite Coatings in *in-vitro* Conditions and Interaction with the Biological Systems

The application of nanostructured V₂O₅ for medical purposes was scarcely studied. There is a lack of comprehensive studies designated to design an efficient V₂O₅ delivery system as well as determine the structure-stability-activity correlation. Because of that, the determination of the impact of the (i) **particle morphology** and (ii) **polymer physicochemical characteristics** on the solubility control of the V₂O₅ within polymer

matrix provided valuable information regarding the design of the V_2O_5 /polymer matrix composites.

When exposed to the PBS medium, just parts of the individual nanowires in the V_2O_5 /PLGA were exposed (Fig. 4.39d) to the surrounding water molecules. As a consequence, the stresses induced by the water molecules were transferred along the polymer matrix, resulting in the prevention of the nanowires' breakage and thus their dissolution. However, a small portion of the nanowires diffused out of the matrix and due to the free exposure to water molecules, they eventually dissolved. Therefore, the success of the V_2O_5 solubility control from the V_2O_5 /PLGA depends on hindering the particles' erosion into the PBS in the early stage of the exposure. V_2O_5 nanowires have a large aspect ratio and are situated almost *in-plane* with the PLGA surface (Fig. 4.39c, d). Their diffusion towards the PBS is greatly hindered and a small portion of them are released from the PLGA matrix, which explains the efficient solubility control of the V_2O_5 nanowires inside the PLGA matrix (Fig. 4.41). In contrast, the sub-micron particles of the commercial V_2O_5 are randomly oriented inside the PLGA matrix and their diffusion from the matrix is not restricted, explaining weaker efficiency of solubility control of the sub-micron V_2O_5 in the PLGA matrix compared to the 1D V_2O_5 nanowires. As a result, a pronounced initial burst effect was observed from the V_2O_5 MPs/PLGA composite (Fig. 4.41). The observed differences among isotropic and 1D particles are the same as in the case of MgO/PLGA polymers, suggesting that better retention of 1D particles in comparison with isotropic particles within PLGA matrix is generally valid for all metal oxides.

The strength of the interaction at the V_2O_5 /polymer interface impacts the diffusion of the V_2O_5 nanowires towards the PBS medium. The IR spectra analysis (Fig. 4.40) confirmed interactions between the C-H group of the polyester and V_2O_5 particles. The inductive effect of the ester group causes generation of the partial positive charge at C-H group (Fig. 5.6). These groups then interact via dipole-dipole interactions with the surface of the V_2O_5 or more precisely with the partially negative oxygen surface atoms involved in vanadyl bonding. The inductive effect is transferring the charge along the whole monomeric units. A monomeric unit of glycolic acid unit possesses five while lactide acid unit possesses eight of both, C-C and C-H bonds, causing the induced partial positive charge to be higher in the case of the PLGA than in the case of the PLA. As a consequence, i) the C-H vibrations bands are overwhelmed in the IR spectra of the V_2O_5 NWs/PLGA, while greatly reduced (but still present) in the IR spectra of the V_2O_5 NWs/PLA (Fig. 4.40b, c) and ii) these interactions are strong enough to retain the nanowires within the PLGA matrix (Fig. 4.39d), while not sufficient enough to retain them within the PLA matrix (Fig. 4.39f). In contrast to the PLA and PLGA, the PS does not contain polar bonds, i.e., there is no charge separation and induction of a partially positive charge (Fig. 5.6). As a consequence, there are no dipole-dipole interphase interactions within the V_2O_5 NWs/PS composites involving C-H bonds and nanowires, which is proved by the absence of any effect on the C-H vibration bands in the IR analysis (Fig. 4.40a).

Apart from the described influence on the surface morphology of the V_2O_5 NWs/polymer composites after exposure to the PBS and IR spectra, the absence/presence of these interactions plays an important role during the preparation procedure for the composites. In the case of PLGA and PLA, dipole-dipole interphase interactions help stabilize the nanowires in the polymer solution by preventing extensive re-agglomeration (Fig. 4.39c, e). Without them the nanowires re-agglomerate like in the case of the V_2O_5 NWs/PS (Fig. 4.39g). As a result, predominantly individual nanowires were released from the PLA surface, while whole bunches of agglomerated nanowires were released from the PS upon exposure to the PBS, making the initial burst effect more pronounced in the case of the PS (Fig. 4.42).

The water-swelling properties of the polymer matrix impacted the diffusion of V_2O_5 NWs towards the PBS. It compromised the efficacy of the chitosan to control the release of the vanadates from the V_2O_5 NWs/Chitosan coating.

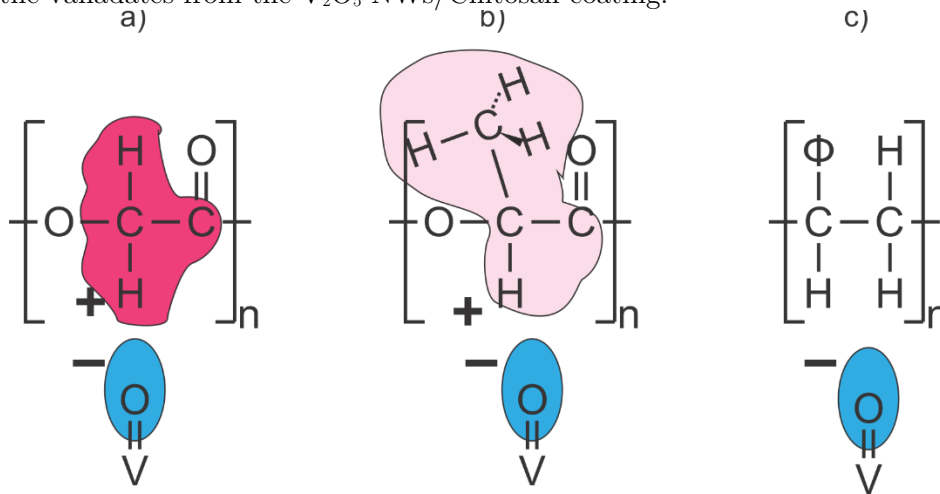


Figure 5.6: Inductive effect of C=O and C-O polyester bonds on C-H bonds and dipole-dipole interactions of a) lactide unit, b) glycolide unit and c) styrene monomeric unit with MgO particles.

The prepared V_2O_5 /PLGA coatings containing 2 % of nanowires provided more sustained release properties than the V_2O_5 /TiO₂ coatings reported in literature [104]. In both cases 1.5 % of the embedded vanadium pentoxide was released within the first day of exposure. However, after 8 days of exposure, 4.5 % of V_2O_5 was released from the PLGA matrix compared to the only 2.5 % from V_2O_5 /TiO₂ within 28 days of exposure.

I exploited the antimicrobial potential of V_2O_5 to protect the PLGA surface against colonization by *S. epidermidis*. The bacteria concentration used in the evaluations of the V_2O_5 /PLGA antibacterial potential was, like in the case of the MgO/PLGA composite coatings, two orders of magnitude higher than proposed by ISO 22196:2007(E). The V_2O_5 was successful in preventing bacteria adhesion at the surface of the PLGA, as the number of colonized bacteria was 70 % lower. In addition, the viability of the attached *S. epidermidis* at the surface of the V_2O_5 /PLGA was reduced when compared to the PLGA surface. Thus, the V_2O_5 exhibited antibacterial protection of the PLGA by both decreasing the number of colonized bacteria and their viability.

Similar to the MgO, I evaluated the impact of the embedded vanadium pentoxide nanowires within PLGA matrix on the red blood cells. The V_2O_5 /PLGA coatings containing 2 % of vanadium pentoxide showed absence of adverse effects on the RBC integrity and morphology. It has been reported that vanadate ions impact the enzymes at nM concentration range [105], while they are toxic to cells at concentrations above 20 μ M [102], [104], [161] as cells are able to reduce the negative impact of vanadate ions by their reduction to vanadyl ions [103]. Further, *in vivo* tests showed improved and accelerated wound healing regarding both, soft and hard tissue repair [109]. I concluded that same levels of released vanadates would not have the same impact on the cellular components, cells in suspension and cells within tissue. Therefore, the reliable evaluation of the cytotoxic potential of the V_2O_5 /PLGA coatings requires development of the new *in vitro* methods, which would as closely as possible resemble involved *in-vivo* processes occurring upon the implantation of such coatings within a human body.

Chapter 6

Conclusions

In this dissertation, the urgent need for multifunctional implant coatings exhibiting both, antibacterial and bioactive properties, was targeted. The thesis was aimed to investigate embedding of MgO and V₂O₅ within MgO/polymer and V₂O₅/polymer composite coatings as an innovative approach for coupling of the antibacterial properties of metal-oxide particles with sustained delivery of dissolved metal ions. I identified (i) **metal oxide particles structural and morphological characteristics**, (ii) **particle agglomeration** and (iii) **metal-oxide/polymer-matrix interphase interactions** as critical parameters to be optimized in the processing of MgO-based and V₂O₅-based multifunctional composite coatings.

The optimization of the metal-oxide antibacterial properties was performed by means of integrated treatment, which included correlation of structural characteristics (surface defects, phase composition) and particle dissolution mechanism to the bactericidal activity and ROS generation potential. A pronounced difference among V₂O₅ and MgO particles was observed. In the case of MgO, the particles with the most potent bactericidal activity were those with the slowest hydration kinetics (and oxide-to-hydroxide transformation) in the physiological solution. Such findings prove competition of water molecules and bacteria for active MgO surface sites when bacteria are exposed to MgO particles in aqueous environment. In the case of the V₂O₅ dissolution of the high aspect ratio nanowires proceeds via a nanowire breakage step, which is an independent process from myeloperoxidase-like catalytic activity. Hydrogen peroxide molecules and water molecules are not competing for the same surface sites of the V₂O₅ nanowires. Thus, the mechanism of particle dissolution must be considered during the optimization of the antibacterial activity of metal-oxide nanoparticles in aqueous environment.

With respect to their ROS generation potential, obtained findings further established MgO and V₂O₅ as a potential antimicrobial agents within the antimicrobial nanostructured materials of next generation. MgO exhibited a non-ROS mechanism of robust antibacterial activity and caused the generation of ROS as a side product of i) MgO hydrolysis or ii) bacteria/MgO interactions. V₂O₅ proved to produce small amount of ROS (not active against bacteria), which were then catalytically transformed in the presence of chloride ions to hypochlorite ions (active against bacteria).

The appropriate choice of metal-oxide particle morphology allowed targeting both, the particle agglomeration and metal-oxide solubility control within the PLGA matrix. 1D particles exhibited reduced agglomeration compared to the isotropic particles, which improved their processing characteristics. On the other hand, the PLGA degradation causes the creation of round-shaped holes at the surface. As a result, the isotropic particles were eroded from the surface. In contrast, 1D particles become just partially exposed to the environment while still situated within the PLGA matrix. As demonstrated, the ability of

partial exposure of the particles at the surface of the PLGA to the media is crucial for (i) a sustained release of the ions in the medium without the initial burst effect and (ii) the retention of the antibacterial activity of the particles within the polymer matrix.

Apart from appropriate particle morphology, the efficient solubility control requires taking into account also metal-oxide/polymer-matrix interphase interactions within the prepared composite coatings. Polymer chemical characteristics were varied and among tested polymers PLGA exhibited the strongest interphase interactions when compared to the PLA, PCL and PS. These interactions were dipole-dipole in nature. When such interactions are not strong enough or not present, 1D particles are rather distributed along the polymer matrix in aggregates. Although most polar, chitosan tends to absorb large water quantities, which proved to be inapplicable in controlled release of inorganic particles without the initial burst effect. Thus, extensive matrix swelling is non-desired in the stabilization of metal oxide particles within polymer-based multifunctional composite coatings where particle retention and sustained ions delivery is required.

To summarize, I have confirmed that integrated design which includes:

- fine tailoring of metal oxide structural and morphological characteristics,
- reduction of particles agglomeration and
- regulation of metal-oxide/polymer matrix interface interactions

resulted in the processing of the MgO/PLGA and V₂O₅/PLGA composite coatings which provided antibacterial protection of the surface against *S. epidermidis* colonization, exhibited sustained release of dissolved ions without the initial burst effect and were not causing neither damage nor morphological changes of the red blood cells. As-prepared composite coatings are promising candidates for further cell testing in *in vitro* and *in vivo* conditions.

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Publications Related to the Thesis

All publications related to the thesis should be referenced in the text.

Journal Articles

- N. Anicic, M. Vukomanovic and D. Suvorov, "The nano-texturing of MgO microrods for antibacterial applications," *RSC Advances*, vol. 6, pp. 102657-102664, 2016.
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Biography

The author of this work obtained International Baccalaureate Diploma at Druga Gimnazija Maribor in 2008. He scored 40/45 points at the Matura exam and won a bronze medal at the International Chemistry Olympiad in 2008. He then studied at the Faculty of Chemistry and Chemical Technology, University of Maribor. As a student, he was awarded with Silver Dean Prize for outstanding student research work. He was also the first coordinator of student tutors as well as a member of several committees at both, the faculty and university. He finished his undergraduate diploma program in 2013 with an average grade 10.0 and was awarded with Henkel Prize for the best diploma thesis of the generation.

In year 2013, he began his doctoral study in the field of Nanosciences and Nanotechnologies at the Jožef Stefan International Postgraduate School and started working as a young researcher at the Advanced Materials department of the Jožef Stefan Institute. During the doctoral study he was awarded for the best oral presentation at YUCOMAT conference, Herceg Novi, Montenegro in year 2014. He also presented his work:

- as an oral presentation at the Annual Meeting of Society of Biomaterials, held in Minneapolis, USA in year 2017;
- as oral presentations at the YUCOMAT conferences held in Herceg Novi, Montenegro in years 2014, 2015 and 2016;
- as oral presentations at the ICMT conference held in Portorož in years 2015 and 2016;
- as an oral presentation at the Conference for Young Scientists in Ceramics, held in Novi Sad, Serbia in year 2015
- as poster presentations at the Young Researchers' day held at IJS in years 2015 and 2016;
- as a poster presentation at the Nano/Medicine Summer school, held in Porto, Portugal in year 2015;

