



Interactions of cells with oxide thin films

Mariya Khokhlova

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THÈSE

Pour obtenir le diplôme de doctorat

Spécialité ASPECTS MOLECULAIRES ET CELLULAIRES DE LA BIOLOGIE

Préparée au sein de l'Université de Caen Normandie

Interactions of cells with oxide thin films.

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**Thèse soutenue publiquement le 04/12/2019
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Abstract

In the present work we demonstrate how oxide thin films can be used as bioactive surfaces, a field of research which is still underexplored. For this purpose, thin films of TiO_2 , Al_2O_3 , VO_x and some others were deposited on glass substrates using the Pulsed Laser Deposition (PLD) technique, and adhesion, proliferation and differentiation of human bone marrow-derived mesenchymal stem cells were evaluated. Cell behavior was analyzed with respect to the various key surface parameters such as chemistry, wettability, morphology and the thickness of films. Our results indicate that thin films of TiO_2 and Al_2O_3 can not only support mesenchymal stem cells adhesion and growth, but also can be used to influence osteogenic and chondrogenic differentiation path. Additionally, effect of oxide thin films on adhesion and growth of cancer cell lines was studied. We showed that culturing these cell lines on thin films affects their growth and, therefore, could be a valuable method to perform screening tests with drugs.

This work will provide a better understanding of correlation between surface chemistry and cellular response, which has a high significance in the field of biomaterials fabrication.

Dans le présent travail, nous montrons comment des couches minces d'oxydes peuvent être utilisées comme surfaces bioactives, un domaine de recherche encore peu exploré. À cet effet, des couches minces de TiO_2 , Al_2O_3 , VO_x et quelques autres oxydes ont été déposés sur des substrats de verre par la technique d'ablation laser pulsé (PLD), et l'adhésion, la prolifération et la différenciation de cellules souches mésenchymateuses humaines dérivées de moelle osseuse ont été évaluées. Le comportement des cellules a été analysé par rapport aux principaux paramètres de surface tels que la chimie, la mouillabilité, la morphologie et l'épaisseur des films. Nos résultats indiquent que les couches minces de TiO_2 et Al_2O_3 peuvent non seulement favoriser l'adhésion et la croissance des cellules souches mésenchymateuses, mais peuvent également être utilisées pour influencer la différenciation ostéogénique et chondrogénique. En outre, l'effet de films minces d'oxydes sur l'adhésion et la croissance de lignées de cellules cancéreuses a été examiné. Nous avons montré que la culture de ces lignées cellulaires sur des films minces affecte leur croissance et, par conséquent, pourrait être une méthode utile pour effectuer des tests de dépistage des drogues.

Cette étude fournira une meilleure compréhension de la corrélation entre la chimie de surface et la réponse cellulaire, qui a un rôle important dans le domaine de la fabrication de biomatériaux.

Keywords: Thin films, Pulsed laser deposition, Surface characterization, Titanium oxide, Aluminium oxide, Vanadium oxide, Biomaterials, Mesenchymal stem cells, Differentiation, Gene expression, Cancer cells

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Contents

1 Introduction and Literature Review	14
1.1 Introduction	15
1.2 Thin films as bioactive coatings	18
1.2.1 Thin films in the development of novel biomaterials	18
1.2.2 Thin films for tissue engineering and stem cell technologies	20
1.2.3 Bioactive metal oxide thin films	22
1.3 Mesenchymal stem cells (MSCs)	25
1.3.1 Application in regenerative medicine and tissue engineering	25
1.3.2 Stem cell adhesion and differentiation	27
1.4 Study of cancer cells behavior	30
1.5 Thin film deposition techniques	31
1.5.1 Chemical deposition techniques	31
1.5.2 Physical deposition techniques	33
<i>References</i>	38
2 Methodology	46
2.1 Preparation of oxide thin films via Pulsed laser deposition	47
2.2 Characterization of deposited thin films	47

2.2.1 X-ray diffraction	47
2.2.2 X-ray reflectivity	48
2.2.3 Atomic force microscopy	49
2.2.4 Water contact angle	50
2.3 Cell culture	51
2.3.1 Human bone-marrow derived mesenchymal stem cells	51
2.3.2 Ovarian cancer cells	52
2.3.3 Chondrosarcoma cells	52
2.4 Study of cell adhesion and proliferation	53
2.4.1 Phase contrast microscopy	53
2.4.2 WST-1 Cell viability and proliferation assay	53
2.4.3 Histological staining	54
2.4.4 Scanning electron microscopy	55
2.4.5 Cell density measurements	56
2.5 Study of human mesenchymal stem cell differentiation	57
2.5.1 Differentiation staining	57
2.5.2 Real-time PCR	59
<i>References</i>	63

3 Results and Discussion 64

3.1 Characterization of deposited thin films	65
--	----

3.1.1 X-Ray diffraction	65
3.1.2 X-Ray reflectivity	67
3.1.3 Atomic force microscopy	69
3.1.4 Water contact angle measurements	71
3.1.5 Energy dispersive X-ray spectrometry	72
3.1.6 Conclusion	73
3.2 hBMSCs adhesion and proliferation on thin films of TiO_2 , Al_2O_3 and VO_x	75
3.2.1 The influence of the parameters of deposition	75
3.2.1.1 <i>Deposition in the O_2 atmosphere</i>	75
3.2.1.2 <i>Effect of different deposition temperature</i>	77
3.2.2 The influence of the thin film composition on cellular adhesion and proliferation	79
3.2.2.1 <i>Cell culture on oxide thin films deposited at different temperatures</i>	79
3.2.2.2 <i>Cell culture on oxide thin films deposited at the same temperature</i>	86
3.2.3 Conclusion	92
3.3 hBMSCs differentiation on thin films of TiO_2 , Al_2O_3 and VO_x	95
3.3.1 Differentiation staining	95
3.3.2 Real-time PCR	98
3.3.3 Conclusion	102
3.4 Influence of metal oxide thin films on the behavior of cancer cells	104
3.4.1 Ovarian cancer cells	104

3.4.2 Chondrosarcoma cells	107
3.4.3 Conclusion	110
<i>References</i>	111
4 Conclusion and perspectives	117

List of Figures

1.1 Applications of thin films in biomedical studies	19
1.2 Micro/nano hierarchical structured surface for enhancing cell response	21
1.3 Multipotency of a mesenchymal stem cell	25
1.4 The triangle of materials refers to variations in chemistry, stiffness, and nanotopography to control the interactions with MSC focal adhesions.	28
1.5 Comparison of division for normal and cancer cells	30
1.6 Schematic of chemical vapor deposition (CVD) reactor	32
1.7 Basic components of DC diode sputtering system	35
1.8 Schematic of PLD system	36
2.1 Information provided by X-ray reflectivity profile	48
2.2 2D schematic of AFM working principle	49
2.3 Contact angle schematic for hydrophobic and hydrophilic surfaces	50
2.4 Schematic of 24-well plate used during in-vitro experiments	51
2.5 Zeiss SUPRA 55 scanning electron microscope	56
2.6 Schematic of PCR process	59

3.1 Temperature-pressure diagram of the vanadium-oxygen system (a). XRD patterns of VO_x thin films deposited at 300 and 500°C. (b). Comparison of XRD patterns of VO_x , TiO_2 and Al_2O_3 thin films deposited at 300°C and glass substrate heated up to the same temperature	66
3.2 XRR curves of TiO_2 films deposited at various temperatures (a) and TiO_2 , Al_2O_3 , VO_x thin films deposited at 300°C (b)	68
3.3 AFM images of surface profiles of glass substrate (a) and VO_x (b), TiO_2 (c), Al_2O_3 (d) thin films deposited at 300°C	70
3.4 Water contact angle measurements for glass substrate (a), glass substrate held at 300°C for 30 minutes, and VO_x (c) TiO_2 (d), Al_2O_3 (e) thin films deposited at 300°C	71
3.5 EDS pattern of TiO_2 (a) and Al_2O_3 (b) thin films deposited at 300 and 200°C, respectively	73
3.6 Cell adhesion after 2 hours of cell culture for controls and VO_x thin films deposited at various temperatures with and without O_2 gas flow	76
3.7 Phase contrast images of MSCs seeded onto VO_x thin films deposited at various temperatures with and without O_2 atmosphere after 24 hours of incubation	76
3.8 Results of WST-1 test for MSCs seeded onto VO_x thin films deposited at 400°C with and without O_2 atmosphere	77
3.9 Phase contrast images of MSCs seeded onto: TiO_2 (top) thin films deposited at 100, 200°C, and at 300°C with different duration of deposition; VO_x thin films deposited at 100, 200 and 300°C (bottom left); Al_2O_3 films deposited at 100°C with different duration of deposition and at 200°C (bottom right)	78

3.10 Optic microscopy images and corresponding cell density after 2 hours (a) and 5 days (b) of cell culture	80
3.11 Cell density vs culture time for cell cultured on glass substrate and TiO_2 , Al_2O_3 thin films (a); corresponding cell doubling time for glass control and TiO_2 , Al_2O_3 thin films (b)	81
3.12 Optic microscopy images of cells on glass control and VO_x thin films after 2 hours (a) and 3 days (b) of cell culture	82
3.13 Phase contrast optical microscopy images of BMMSCs on glass control (a, d, g) , TiO_2 and Al_2O_3 thin films (b, e, h and c, f, i, respectively) after 6, 16 and 26 days of cell culture	83
3.14 Phase contrast images of MSCs seeded onto VO_x thin films deposited at various temperatures with and without O_2 atmosphere after 2 days of incubation	84
3.15 SEM images of BMMSCs on glass control, TiO_2 and Al_2O_3 thin films after 14 days of culture (a), SEM images of multicellular structures on Al_2O_3 (b)	85
3.16 Optic microscopy images of BMMSCs on glass control, TiO_2 and Al_2O_3 thin films after two hours of cell culture (a); corresponding cell density (b)	87
3.17 Cell density BMMSCs cultured on glass control, TiO_2 and Al_2O_3 thin films in the initial point of cell culture and after 2, 4 and 7 days (a); initial point and after 4 and 7 days of cell culture (b)	88
3.18 Phase contrast optical microscopy images of Van Gieson (a), hematoxylin phloxine saffron (b) and Masson's trichrome(c) staining after 14 days of cell culture on glass control, TiO_2 and Al_2O_3 -coated substrates	89
3.19 SEM images of glass control (a,d), TiO_2 (b,e) and Al_2O_3 -coated (c, f) substrates after 7 days (top) and 14 days of cell culture (bottom)	90

3.20 Cell density of hBMMSCs cultured on glass control and VO_x thin films after 2 hours of culture (a) Phase contrast images of hBMMSCs on same substrates after 10 days of culture with visible difference in amount of cells (b)	91
3.21 SEM images of glass control (a,c) and VO_x -coated (b, d) substrates after 7 days of cell culture with magnification of $\times 1000$ (top) and $\times 5000$ (bottom).	91
3.22 Phase contrast images of MSCs after 4 days of culture on CuO, MgO and ZnO thin films deposited at 100°C (a), SEM images of cells grown on CuO thin films (b)	93
3.23 Alizarin red staining of (a) MSCs seeded on glass control, TiO_2 and Al_2O_3 -coated substrates after 14 days of cell culture and (b) MSCs seeded on glass control and VO_x in separate experiment	96
3.24 Absorbance of the eluted Alcian blue for MSCs seeded on glass control, VO_x , TiO_2 and Al_2O_3 -coated substrates after 14 days of cell culture	97
3.25 Oil red staining of MSCs seeded on glass control, TiO_2 , Al_2O_3 and VO_x -coated substrates after 14 days of cell culture	98
3.26 Osteogenic differentiation of hBMMSCs on the various samples and real-time PCR analysis of osteogenesis-related genes after culturing for 14 days	99
3.27 Chondrogenic differentiation of hBMMSCs on the various samples and real-time PCR analysis chondrogenesis-related genes after culturing for 14 days	101
3.28 Comparison of relative expression of ITGA1 and ITGB1 after cell culture in standard α -MEM (a) and chondrogenic media (b)	102
3.29 IGROV1-R10 on plastic, glass control, VO_x -, CuO- and ZnO-coated substrates	105

3.30 Cell Tox Green tests results for IGROV1-R10 seeded on plastic, glass control, VO _x -, CuO- and ZnO-coated substrates	105
3.31 SKOV3 on plastic, glass control, VO _x -, CuO- and ZnO-coated substrates	106
3.32 Cell Tox Green tests results for SKVO3 seeded on plastic, glass control, VO _x -, CuO- and ZnO-coated substrates	107
3.33 JJ012 cells on plastic, Al ₂ O ₃ -, TiO ₂ -, CuO-, ZnO- and VO _x -coated substrates	108
3.34 SW1353 cells on plastic, Al ₂ O ₃ -, TiO ₂ -, CuO-, ZnO-, VO _x -coated substrates	108
3.35 FS090 cells on plastic, Al ₂ O ₃ -, TiO ₂ -, CuO-, ZnO- and VO _x -coated substrates	109
3.36 CH2879 cells on plastic, Al ₂ O ₃ -, TiO ₂ -, CuO-, ZnO- and VO _x -coated substrates	109

List of Tables

Table 1.1 Overview of oxide thin films that have been studied in terms of their biological activity. *PET - polyethylene terephthalate, SS - stainless steel	24
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Table 2.1 Primer sequences used for PCR	62
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List of abbreviations

AFM	Atomic Force Microscopy
α -MEM	alpha Minimum Essential Medium
BMSCs	Bone Marrow Stem Cells
CVD	Chemical Vapor Deposition
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EDS	Energy-Dispersive X-ray Spectroscopy
FGF	Fibroblast Growth Factor
H ₂ O _d	Distilled water
HA	Hydroxyapatite
hBM MSCs	Human Bone Marrow-derived Mesenchymal Stem Cells
MBE	Molecular-Beam Epitaxy
MSCs	Mesenchymal Stem Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PLD	Pulsed Laser Deposition
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SEM	Scanning Electron Microscopy
WST	Water-Soluble Tetrazolium
XPS	X-ray Photoelectron Spectroscopy
XRD	X-Ray Diffraction
XRR	X-Ray Reflectivity

1 Introduction and Literature Review

1.1 Introduction

Over the past decades, there has been a growing interest in increasing the quality and expectancy of life. One of the ways to reach this goal is to develop materials to replace damaged organs and tissues. Dozens of different materials (polymers, ceramics and metals) were used to create artificial joints, organs and tissues, including bone and skin tissues, nerve fibers and blood vessels. Fabrication of biomaterials is pronounced interdisciplinary field, which uses achievements of physics, chemistry, medicine, electronics and other disciplines, and there is an ongoing search for new approaches to improve biomaterials performance.

Results of numerous studies have demonstrated that surface properties of biomaterials are of great importance for directing response of biological matter [1, 2]. One of the common ways of surface modification is a deposition of a thin film on a suitable substrate. Metal oxide coatings have attracted attention in the field of biomaterials fabrication as some of them are known to be bioactive [3, 4], but this research field remains unexplored. Most studies of the potentialities of oxide thin films as scaffolds for cell growth dealt with titania surfaces, either rough or patterned [5]. Various techniques, such as magnetron sputtering or dip coating, have thus been used for a titanium dioxide deposition. It has been shown that titania coatings not only have an antimicrobial activity, but also can enhance cell adhesion and proliferation [6–8].

The determining factors to identify the potential of the material include its thermodynamic stability, resistance to corrosion, and, of course, its non-toxicity and the fact that it does not induce allergic reactions. Actual state of the art suggests that the surface topography is the determining parameter affecting cell behavior and differentiation through interactions with cell surface adhesion proteins, such as integrins, that modulate cell adhesion, gene expression and overall cell behavior [9]. At the microscale level, surface features that are of the same order of magnitude as the cell only play the role of contact guidance for the whole cell orientation. The important structuration for the cell fate lies at the nanoscale, with elements that are of the same order of size as the cell receptor. Most of the studies

of cell growth on oxide thin films have dealt with model surfaces, prepared by standard top-down micro/nano microelectronic fabrication techniques (typical surfaces covered with pillars, with systematic variation of size, dimensions and pitches). On the one hand the drawbacks are the high cost of these techniques, difficulty to scale up to large production and optimization for a limited number of materials, and on the other hand, the resulting surfaces lack irregularity that seems beneficial to cell adhesion. Alternatively, it has been tried to produce disordered surfaces with micro/nano topography with altering surface techniques (polishing, etching, plating, anodizing, etc.), but while cell adherence was improved, results were difficult to understand due to the lack of reproducibility. To improve such engineering methods, the development of new functional biomaterials is clearly necessary.

Oxide thin films, that are usually utilized for their electronic properties [10], are an attractive option, since the control and analysis of the surface are simplified. However, a very limited number of oxides have been studied. Besides TiO_2 , investigations were done on ZnO , ZrO_2 and a very few other oxides. Furthermore, mechanisms of the cell-material interaction are still not completely clear. Thus, the idea of the present work is to minimize influence of various parameters on cellular response except surface chemistry, which can be achieved thanks to the very low values of surface roughness (0.1 - 0.2 nm) of thin films produced by Pulsed Laser Deposition (PLD) technique.

The main objectives of this study can be summarized as follows:

- to identify biocompatible metal oxide thin films,
- to evaluate adhesion and proliferation of cells,
- to study of the effect of composition and other parameters on cell differentiation.

Within this work, TiO_2 , Al_2O_3 , VO_x and some other oxide thin films are prepared and

deposited on glass substrates using pulsed laser deposition method. Human mesenchymal stem cells derived from bone marrow were cultured on the surfaces of the thin films and their adhesion, growth and differentiation further evaluated. In addition, adhesion and proliferation of ovarian and chondrosarcoma cancer cells upon interaction with oxide thin films were studied. Our results illustrate that having great diversity of compositions and intrinsic physical properties, metal oxide thin films represent a very interesting choice as substrates for cell cultures in researches related to cell-material interaction. We believe that this study will demonstrate the feasibility of using metal oxide thin films for investigations concerning behavior of various cell types.

1.2 Thin films as bioactive coatings

1.2.1 Thin films in the development of novel biomaterials

With the rapid technological progress and ongoing efforts to improve the quality and expectancy of life, as well as existing clinical treatments, there is a growing demand for fabrication and investigation of new biomaterials. One of the essential concepts in regards to biomaterials is their biocompatibility. Biocompatible materials are defined as materials that are able to integrate into the patient's body (host) with producing an appropriate response from the biological tissue [11]. Among the areas of biomaterials application, there are three main ones, namely:

- orthopedic implants, such as implants for bones fracture fixation, joint replacement, etc;
- dental restorative materials, which include ceramics, removable and fixed prosthetics, metal and composite filling materials and others;
- cardiovascular implants, such as cardiac blood vessels, coronary stents, prosthetic heart valves, as well as some other materials [12–14].

According to the regulatory requirements, there are several important properties a biomaterial must possess. Biomaterials should not cause any allergic, toxic and cancerogenic effects on the body, as well as development of any local inflammatory reaction or infection. Retaining their functional properties for the intended period of exploitation is another property each biomaterial is supposed to have [15]. Biomaterials currently used do not always meet all the necessary requirements. For example, bioinert materials with sufficient mechanical strength often have poor integration into tissues. In turn, materials with high bioactivity and compatibility can have poor mechanical properties. This problem leads to the search for new approaches and materials for coatings.

Successful application of the bioactive material strongly depends on how well it integrates in the host tissue. In turn, integration of the material is directly related to its surface prop-

erties, since the biological matter upon contact immediately interacts with the uppermost layers of materials. Essential biological processes such as protein adsorption and cell adhesion are also surface phenomena. Therefore, changing surface chemical and/or chemical properties is the obvious way to influence performance of the biomaterial. One of the most common ways to modify the surface characteristics and its biological response is to deposit a thin film.

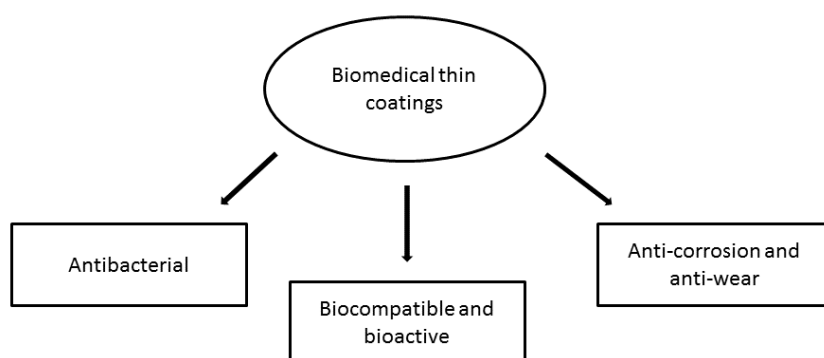


Figure 1.1. Applications of thin films in biomedical studies. They are used as coatings that influence bioactivity, antibacterial and mechanical properties of the biomaterial.

Besides their ability to improve biocompatibility of the material, thin film coatings are of great interest because they can serve as inhibitors of potentially harmful ion release, as well as they significantly improve mechanical stability and strength of the material (Fig. 1.1). Moreover, thin films are extensively studied as the coatings in research concerning stem cell biology and development of new approaches for cell-based therapies. For example, it has been demonstrated that thin films can be used as efficient platforms for stem cell growth and differentiation, engineering of cell surfaces and other applications [16, 17].

1.2.2 Thin films for tissue engineering and stem cell technologies

Various materials in the form of deposited coating were studied in terms of their application in biomedicine. A few types of materials that attracted great attention in this field will be discussed below.

Hydroxyapatite (HA). HA coatings are broadly used to improve the osseointegration of implants. Being an analogue of the main component of the bone mineral base, hydroxyapatite has strong osteoconductive properties, provides adhesion of proteins and bone tissue cells, is actively involved in ion exchange and metabolism of the bone matrix, supports ionic and covalent bonds with bone minerals [18]. Creating optimal conditions for osteoconduction, hydroxyapatite at the same time undergoes osteoclastic resorption, dissolves in a liquid medium and is absorbed within 6-10 months. In the form of thin coating, modified HA showed promising results for osteoblasts proliferation and supporting mesenchymal stem cells adhesion and osteogenic differentiation ability [19, 20].

Bioglasses and non-metal oxides. Biocompatible glass include: SiO_2 (40-60%), P_2O_5 (6% in average), CaO (10-32%) and Na_2O (about 25%) [21]. Biological activity of biocompatible glass is based on the chemical degradation (solubility) of these materials surface in a liquid biological media. As a result, calcium ions and phosphorus compounds come to the surface, what promotes the formation of apatite crystals on the material. These crystals form the centers of osteoid mineralization and provide a physicochemical bond between the bone matrix and the surface of the material. Thanks to these properties, bioglass coatings are widely studied and used to improve biocompatibility of implants [22]. Besides being part of the bioglass composition, SiO_2 thin films were recently used in order to study the effect of microtopography on the osteogenic differentiation of mesenchymal stem cells [23]. Graphene oxide is another non-metal oxide that demonstrates big promise for biomedical application, namely for anticancer therapies [24].

Nanomaterials. Among the coating materials with great potential for use in biomedicine, nanomaterials are extensively studied thanks to their exceptional properties. Characterized

by nanoscale particle size, they have large surface-to-volume ratio, ability to diffuse across cell membranes, adsorb onto tissues and penetrate them [25]. As known, cellular response is greatly affected by such film surface properties as wettability, roughness and morphology [26, 27]. Having a nanoscale size of surface features, nanomaterial coatings provide an optimal topography for enhanced cell-material interaction (Fig. 1.2). Moreover, they can significantly influence cell proliferation and differentiation [28]. Nevertheless, as any other materials, nanomaterials have their own drawbacks. For example, small deviation in the size of nanoparticles can lead to a significant change in the bio-response of material [29], which means that this deviation in some cases causes drastic increase in material toxicity. Application of such materials can be potentially dangerous as it is often complicated to control and reproduce nanoparticle size. Therefore, it is necessary to further investigate long-term health effects and reproducibility of the synthesis [30].

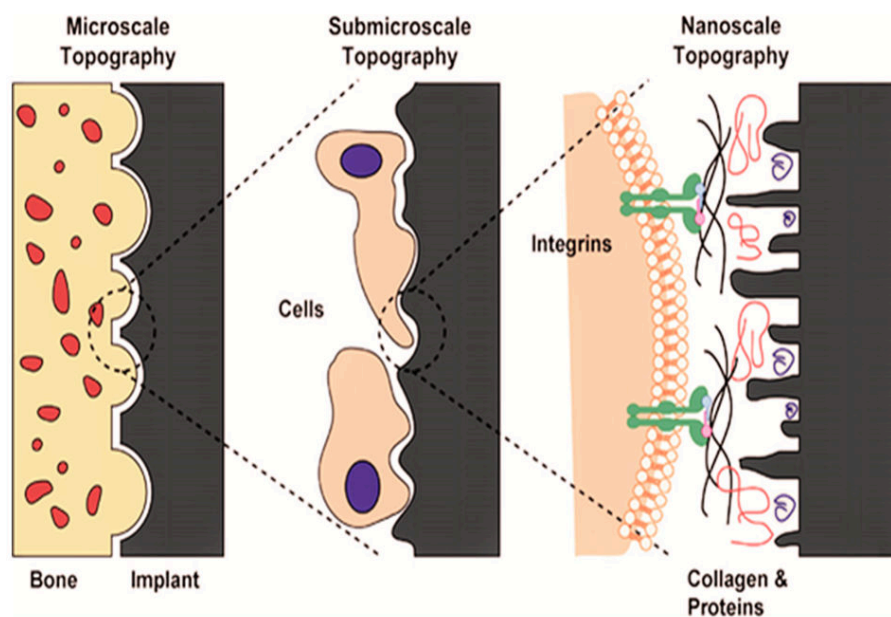


Figure 1.2. Micro/nano hierarchical structured surface for enhancing cell response. Nanoscale topography activate cell adhesion proteins, integrins, thus facilitating cell-material interaction [31].

As a promising alternative for nanoparticles and conventionally utilized organic coatings,

oxide thin films gained much attention thanks to their known bioactivity, almost unlimited variety of compositions and intrinsic physical properties. Actively studied due to their remarkable electronic and magnetic properties [32, 33], majority of oxide thin films, however, have not been exploited as potentially bioactive materials.

1.2.3 Bioactive metal oxide thin films

Metallic elements are abundant and can form stable oxides of great diversity of composition and properties. As it is mentioned above, many metal oxides have shown promising results in the field of biological studies. Large part of these studies is concentrated on utilizing metal oxides in the form of nanoparticles. For example, antibacterial activity of ZnO nanoparticles has been demonstrated in several studies [34, 35]. For instance, Wahab et al. demonstrated results showing positive effect of ZnO nanoparticles on reduction of bacteria proliferation [35]. Having their distinctive advantages, nanoparticles, in comparison to thin films, are generally more complicated to characterize and reproduce their surface properties.

One of the first oxide thin films application that attracted attention of researchers was improving prosthetic implant materials. For example, implants made of titanium (Ti) and its alloys are widely used due to its biocompatibility and good mechanical properties. In order to minimize the probability of the implant failure, it is important to prevent formation of bacterial biofilm on its surface and to ensure appropriate biological interaction between implant and surrounding tissue. Adsorption of oxygen atoms from air leads to the spontaneous formation of the passive oxide layer at the surface of Ti materials. In case of bone replacement, bio-inert nature of this layer impedes good contact between living tissue and the implant, thus leading to osseointegration declining. Moreover, formation of the niche around implant increases the probability of bacteria cell adhesion and inflammation. Besides hydroxyapatite coatings mentioned above, metal oxide thin coating deposition has been one of the strategies to overcome these problems.

For example, TiO₂ thin films attracted great attention thanks to their photocatalytic

activity. Reactive oxygen species (ROS), formed upon UV irradiation, prohibit bacterial colonization and enhance osseointegration of Ti-based implant [36]. Numerous studies have demonstrated bioactivity of TiO_2 and that these coatings not only improve antimicrobial activity of the material, but also its chemical stability [37–44]. TiO_2 thin films are also considered to be osteoconductive and show superior osteoblast adhesion and proliferation compared to uncoated substrates [45, 46].

Other oxide thin films that have been studied quite actively are CuO , ZnO and ZrO_2 . They are known to have antibacterial properties and thin films of these oxides showed promising results for preventing biofilms formations [47–50]. Deposition of ZnO and ZrO_2 coatings has been also employed to investigate biocompatibility and osteogenic activity of these oxides [51–53]. For example, Trino et al. in [51] concluded that functionalized ZnO thin films exhibit properties favorable for osseointegration and mechanical characteristics of the implants. Furthermore, antibacterial activity and impact on cellular behavior of Al_2O_3 thin films were also assessed in multiple studies where the results illustrated potential of this oxide in biomedical applications, such as cell therapy and orthopedics [54–56].

In summary, taking into account the diversity of oxide film compositions, number of metal oxides that have been studied in terms of their biological activity is rather limited (Table 1.1). As a consequence, it creates a space for numerous investigations aiming to improve existing biomaterials and biomedical technologies.

Table 1.1. Overview of oxide thin films that have been studied in terms of their biological activity. *PET - polyethylene terephthalate, SS - stainless steel.

Thin film composition	Substrate	Method	Cell/bacteria/virus type	Reference
TiO₂	Silicon	Magnetron sputtering	<i>Osteoblasts</i>	[6]
	SS*	Dip coating	<i>Bacillus pumilus</i>	[7]
	Titanium	Slow-rate sputter deposition	<i>Osteoblasts</i>	[8]
	Glass	Chemical vapor deposition	<i>Bacteriophage T4</i> <i>Escherichia coli</i>	[37]
	Glass	Magnetron sputtering	<i>Epithelial-like cells</i>	[38]
	Silicon	Magnetron sputtering	<i>Primary rat osteoblasts</i>	[39]
	Titanium	Cathodic arc deposition	<i>Osteoblast-like cells</i>	[40]
	Titanium	Chemical vapor deposition	<i>Fibroblasts</i>	[41]
	Glass	Dip coating	<i>Mesenchymal stem cell</i>	[42]
	Glass	Magnetron sputtering	<i>Osteoblast-like cells</i>	[43]
	Glass	Sol-gel	<i>Fibroblasts</i>	[44]
	Glass	Sol-gel	<i>Osteoblasts</i>	[45]
	Titanium	Atomic layer deposition	<i>Fibroblasts</i> <i>Osteoblasts</i> <i>Staphylococcus aureus</i> <i>Escherichia coli</i>	[46]
CuO	Copper	Solution chemistry	<i>Staphylococcus aureus</i> <i>Escherichia coli</i>	[47]
ZnO	PET*	Magnetron sputtering	<i>Escherichia coli</i>	[48]
	Silicon	Pulsed laser deposition	<i>Escherichia coli</i>	[49]
	Titanium	Solution processing	<i>Mesenchymal stem cell</i>	[51]
ZrO₂	SS*	Pulsed laser deposition	<i>Staphylococcus aureus</i> <i>Escherichia coli</i>	[50]
	Glass	Sol-gel	<i>Mesenchymal stem cell</i>	[52]
	Titanium	Pulsed plasma deposition	<i>Mesenchymal stem cell</i> <i>Pre-osteoblasts</i>	[53]
Al₂O₃	Al alloy	Micro-arc oxidation	<i>Mesenchymal stem cell</i> <i>Staphylococcus aureus</i> <i>Escherichia coli</i>	[54]
	Glass	Magnetron sputtering	<i>Mesenchymal stem cell</i>	[55]
	Silicon	Pulsed laser deposition	<i>Osteoblast-like cells</i>	[56]

1.3 Mesenchymal stem cells (MSCs)

1.3.1 Application in regenerative medicine and tissue engineering

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that have self-renewal properties and ability to differentiate into various cell types that form specific tissues and organs in the human body including fibroblasts, chondrocytes, osteoblasts, myoblasts and adipocytes (Fig. 1.3).

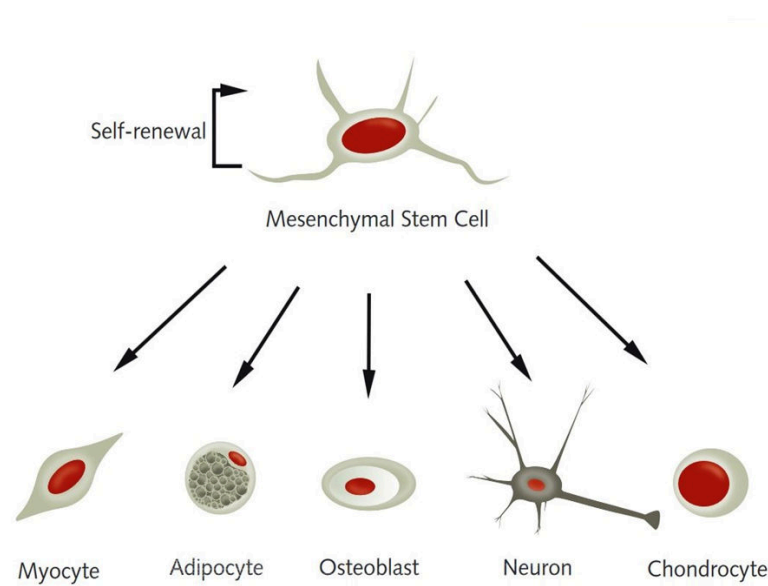


Figure 1.3. Multipotency of a mesenchymal stem cell: ability to differentiate into cells of muscle tissue (myocytes), fat cells (adipocytes), bone tissue (osteoblasts), nerve cells (neurons) and cells of cartilage (chondrocytes) [57].

Moreover, MSCs are capable to fight apoptosis (cell death), promote angiogenesis (formation of new blood vessels) and have antimicrobial and anti-inflammatory effects [58]. Thanks to these remarkable properties mentioned above, MSCs attract great attention in such fields as regenerative medicine and tissue engineering [59]. While the aim of both these disciplines is to repair damaged organs or tissues and to restore their normal functions, unlike regenerative medicine, in tissue engineering healthy tissue is developed *in vitro*, i.e. outside of the

living organism. For *in vivo* tissue engineering applications, various scaffolds, both synthetic and natural, can be used to deliver MSCs to the site of tissue damage or injury [60].

Another major advantage of using MSCs in cell-based therapies, it is the possibility to derive them autologously, which means using individual's own stem cells. In this case the risk of inflammation and rejection of implanted material is significantly reduced [61]. Autologous MSCs can be obtained from different sources including dental tissues, liver and adipose tissue. Stem cells derived from adipose tissue are widely studied for the therapeutic use thanks to the ability to differentiate into numerous lineages and their immunosuppressive properties [62]. Another, much more common way to get autologous MSCs is to harvest them from a bone marrow. Autologous bone-marrow derived stem cells demonstrate a great potential for cell-based therapies for more than 50 years. Besides self-renewal, bone marrow stem cells (BMSCs) also have remarkable, almost unlimited, capacity of developing into tissue- or organ-specific cell types including osteoblasts, adipocytes, neural and blood cells, cardiac myocytes and many others [63].

One of the biggest obstacles to use and study MSCs for cell therapies and treatments is their relatively small amount. The stem cell number needed for the clinical trials can reach values of hundreds of millions, whereas, for example, for the newborns, the amount of MSCs was roughly estimated to be close to 1 per 10000 marrow cells. Moreover, the number of stem cells is greatly decreasing with age of the patients, and in comparison to the newborn, there is a two orders of magnitude decrease in MSCs relative amount for elderly patients [64]. Quality of MSCs, i.e. their capacity for long term self-renewal, proliferation and differentiation, is also declining with age. Therefore, it is highly important to develop new methods to significantly increase the rate of expansion of MSCs during the *in vitro* experiments.

1.3.2 Stem cell adhesion and differentiation

Cell adhesion is the process of cell attachment to the surface or to the extracellular matrix of another cell. One of the key stages of this process is the interaction of specific plasma membrane glycoproteins and extracellular matrix. Negatively charged glycoproteins are located on the outer part of the cell membrane, while their polypeptide chains penetrate the cell membrane and come into contact with intracellular proteins. Thus, these cell membrane glycoproteins play a key role in intercellular recognition and cell adhesion. One of the most important proteins involved in the adhesion process is fibronectin [65]. This glycoprotein consisting of two identical disulfide bound polypeptide chains can be found circulating in the blood plasma or located on the surface of such cells as fibroblasts. Another important family of proteins that mediate cell adhesion are integrins. These transmembrane receptors have the α - β heterodimeric structure and participate in the formation of the contact between cell and extracellular matrix [66].

For biomaterials fabrication, the understanding of this complex process plays a fundamental role as it is the initial stage of the interaction between biological matter and the material. Cell adhesion to the biomaterial can undergo through direct and indirect way. In the latter one, proteins of cell membrane interact not directly with the material, but with proteins that were previously adsorbed onto material surface [67]. Surface roughness and wettability are among properties that affect cell adhesion the most. Higher values of roughness mean that surface have a lot of points that will serve as anchor sites for cell membrane proteins. In turn, high wettability favor protein adsorption and, consequently, leads to enhanced cell adhesion, as well as proliferation.

Stem cell differentiation. In the studies of therapies employing stem cells, it is crucial to control differentiation. For various clinical applications, it is necessary to direct cell differentiation in a certain way depending on whether the blood vessel is to be repaired, epithelial tissue to be formed, etc. Stem cell differentiation path can be influenced by a wide range of factors, such as presence of a specific protein or particular physical property of the

substrate [68, 69]. It has been demonstrated that stem cell fate can be as well defined by confining and controlling the shape of the cell [70]. Furthermore, cell differentiation can be induced by surface chemistry [71].

Summing up, stem cell can respond to any changes in surface property, for example, chemistry or topography (Fig. 1.4). Surrounding environment define stem cell fate, adhesion and spreading, capacity for self-renewal and other important processes.

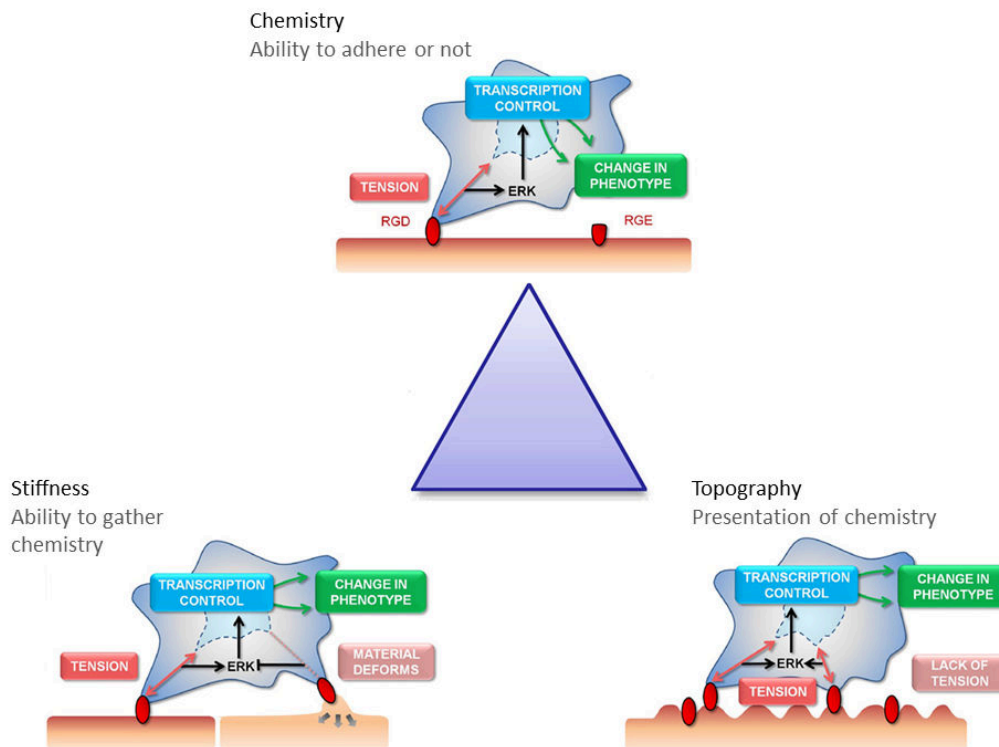


Figure 1.4. The 'triangle' of materials refers to variations in chemistry, stiffness, and nanotopography to control the interactions with MSC focal adhesions. The three cartoon panels show MSC adhesion to chemical, stiffness, and topographically modified surfaces and how this influences stem cell tension and signaling and, hence, subsequent differentiation and phenotype. ERK refers to extracellular signal-regulated kinase, RGD - characteristic motif in the fibronectin sequence, and is a tripeptide consisting of arginine, glycine and aspartate, RGE - same, except aspartic acid replaced with glutamic acid [72].

Up to the present, the mechanism of MSCs differentiation is not fully clear. Accordingly, there are numerous studies that are aiming to find and determine key factors affecting cell

differentiation path. Taking into account all the above, it is thus important to continue exploring the mechanisms lying in the processes of cell adhesion and differentiation as they remain not completely understood.

1.4 Study of cancer cells behavior

Sometimes, cells become cancerous when a series of mutations occur. These mutations happen in various genes that regulate cell proliferation and usually are either inherited or are more often caused by carcinogens in our environment. As a result, unlike in case of normal cells, multiplication and division of cancer cells undergo in an unregulated way with replication of abnormal and damaged cells (Fig. 1.5). Another difference consists in the ability of cancer cells to penetrate into nearby tissues, as well as circulate and spread freely in the remote areas of the body [73].

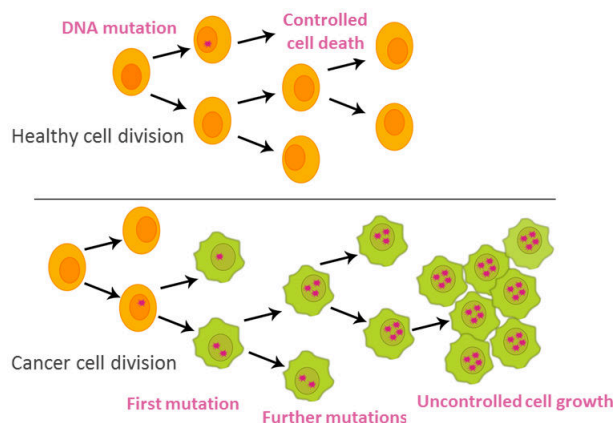


Figure 1.5. Comparison of division for normal and cancer cells. All damaged normal cells are programmed for apoptosis (cell death), while in case of cancer cells, they continue to divide in uncontrolled way. The figure adapted from [74].

Despite the tremendous progress made in cancer research over past years, the understanding of how to control and cure this disease is yet to be improved. Within this study, several cancer cell lines were used for experiments concerning their interaction with metal oxide thin films. One of them being ovarian cancer cell lines. Ovarian cancer is considered as one of the most common cancer types among women and has a high ranking as cause of death from cancer [75]. Another type of cancer cells that were used are cells derived from chondrosarcoma, which is malignant tumor composed predominantly of cells producing cartilage [76].

1.5 Thin film deposition techniques

For the last decades, thin films had a great impact on the variety of applications. They are used for optic and energy storage devices, and in areas like nanolithography, hardening coating, thermoelectricity and many others [77, 78]. Having the thickness varied from a few nanometers to several micrometers, thin films are of great interest for material science nowadays, as in modern technologies it is very important to minimize the size of the material and to make devices as light and compact as possible. Another reason why thin films attract attention is that they have some properties that can be absent in the bulk materials. While parameters such as crystallographic defects or roughness likely will have no effect on the bulk material properties, in some cases they can be a key factor that will dramatically influence thin film property of the same composition. Therefore, the control of thickness, homogeneity, morphology and crystallinity is crucial for the thin films production. There are various methods used in order to synthesize a thin film, each having their advantages and disadvantages. Commonly, they are divided into physical and chemical deposition techniques.

In this subchapter, the main principles of the methods used for deposition of thin films for various applications, including those related to biomaterial fabrication, will be described. As a technique that has been used during my study, the process of pulsed laser deposition (PLD) will be discussed more in depth, while experimental details will be provided in the following chapter.

1.5.1 Chemical deposition techniques

The main principle of chemical methods is a deposition of precursors in gaseous or liquid phase on the substrate as a result of a chemical reaction. Sol-gel and chemical vapor deposition methods will be further discussed.

Sol-gel techniques. Sol-gel process is based on the dissolving organometallic salts, metal

alkoxides, salts of inorganic acids into solvents with a subsequent formation of a sol and its conversion to a gel. Afterwards, there are steps of drying and crystallization of the well-adhered dense coating on the substrate [79]. Thin films prepared via sol-gel route are extensively used to improve material corrosion and wear resistance; they are also used for bioactive surfaces and as antireflective coatings [80, 81]. Coatings synthesized by means of sol-gel method served as surfaces to assess cytotoxicity of a material and its biocompatibility [82, 83]. Environmentally friendly, this technique allows coverage of materials with complex shape and structure and does not require expensive, complicated equipment and provides a good control of microstructure and chemical composition of final product. Due to the good mixing of initial components in the liquid phase on the atomic level, as well as processing at mild temperatures, sol-gel derived coatings have also a high level of homogeneity. However, there are some disadvantages, such as length of the process and high cost and toxicity of initial materials in some cases.

Chemical vapor deposition. In the CVD process, the deposition of thin films is carried out through the reaction of volatile precursor components at the substrate with subsequent formation of the solid phase film. Precursors are evaporated thermally and after their mixing with the inert gas flow, they are transferred with a certain speed to the reactor [84].

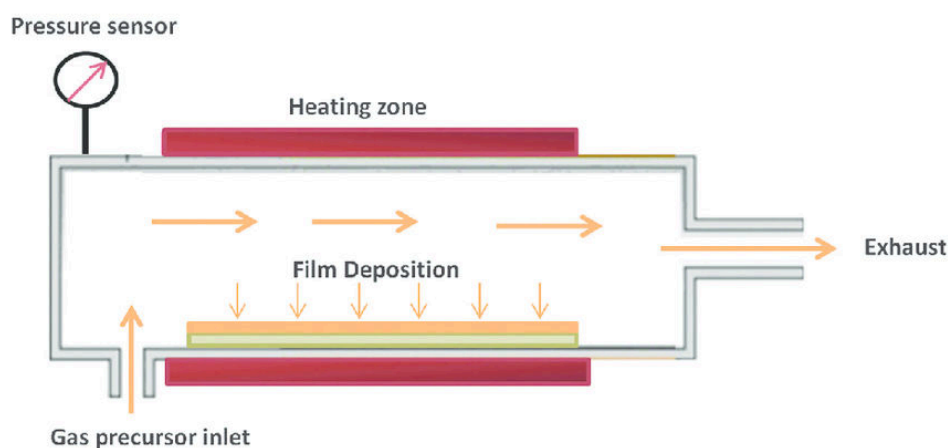


Figure 1.6. Schematic of chemical vapor deposition (CVD) reactor. Films are deposited on samples or substrates after high-temperature interaction and/or reactions of gaseous precursors [85].

This technique is used to fabricate films with uniform composition and thickness even on the substrates with a complex shape. In the industry, CVD is used in the production of compound semiconductors, glass coatings and silicon devices [86]. In regards to scientific studies, thanks to the fact that it allows using great variety of initial components with a high level of reproducibility of the films properties, CVD technique is widely used as well [87–89]. In regards to investigations related to biological matter, for example, this method was used to deposit thin films to study behavior of stem cells and antibacterial materials [90, 91].

Chemical bath deposition. Known from 1869, this method, also called chemical solution deposition, is considered as one of the oldest and simplest way to deposit a thin film. This method of film growth is straightforward and based on the immersion of the substrate in the precursor liquid solution, which leads to the following formation of the solid phase by the chemical reactions [92]. This technique does not require expensive and complex equipment, and allows fabrication of batches with large surface area. Providing controlled precipitation, it is broadly used to synthesize chalcogenide coatings and many types of oxide thin films [93–96].

1.5.2 Physical deposition techniques

Physical methods are widely studied and some of them are the most frequently used methods for thin film deposition. Fundamentally, they all based on a few basic steps, such as transport of material in its atomic form from the target to substrate, followed by its condensation, thus forming the coating. Among physical deposition techniques, evaporation and sputtering methods can be distinguished. All evaporation techniques include thermal evaporation of atoms of solid state target that are transferred to the substrate in the gas phase thus formed. All these processes are carried out under controlled atmosphere or vacuum.

Molecular beam epitaxy. The main principle of MBE is the interaction of atomic and

molecular beams on the surface of the heated substrate. Elementary components of the desired coating are evaporated and supplied separately under ultra-high vacuum [97]. The major disadvantage of this method is the uncertainty of stoichiometry in the final product and a rather low rate of the deposition. However, with MBE, it is possible to make a deposition at low temperatures and to monitor the growth process very carefully. Thanks to these benefits, MBE-deposited films are being studied for the use in optoelectronics and semiconductor devices [98].

Cathodic arc deposition. In this plasma-based film growth method a thin layer is formed on the substrate after condensation of a cathode target material that was evaporated with the use of the electric arc [99]. Main drawback of this process is the presence of microdroplets in the plasma stream that have to be filtered; otherwise, they degrade the homogeneity of the coating. Nevertheless, this technique provides a quiet fast and well controlled way to produce protective, wear resistant and decorative coatings of high quality [100]. Cathodic arc deposition is also used to produce thin coatings for investigations related to the bioactivity of materials, such as, for example, the effect of titania and zirconia surfaces on osteoblasts-like cells behavior or toxicity of titanium nitride coatings [101, 102].

Vacuum thermal evaporation. This physical vapor deposition method is a low-cost technique that considered being one of the simplest ways to fabricate a thin film. In this case the deposition process includes thermal evaporation of the source material inside the vacuum chamber and its subsequent condensation on the surface of the substrate [103]. Thus prepared coatings can be used for phase change memory applications and solar cells [104, 105].

Besides techniques described above, physical deposition methods include sputtering techniques, such as, for example, direct current (DC) and radio frequency (RF) magnetron sputtering (Fig. 1.7).

Sputtering deposition. It is one of the most frequently used method in which a thin film deposition involves the bombardment of the cathode target by the high energy beam accelerated in the electrical field. Then, thus sputtered particles condense on the substrate placed in the proximity to the negatively charged target [106].

To enable the sputtering of insulating materials radio frequency (RF) power supply was developed, while direct current (DC) sputtering is used for conductive materials, such as, for example, doped semiconductors or metals [108]. Magnetron sputtering deposition yields adherent, dense and stable films and it is used in the industry to fabricate coatings for protection, as well as coatings with specific electrical and optical properties [109, 110]. There are many studies with experiments related to bioactivity of thin films deposited by sputtering techniques. For example, radio frequency magnetron sputtering was used to prepare carbonated hydroxylapatite thin films in order to assess their effect on differentiation of mesenchymal stem cells [111].

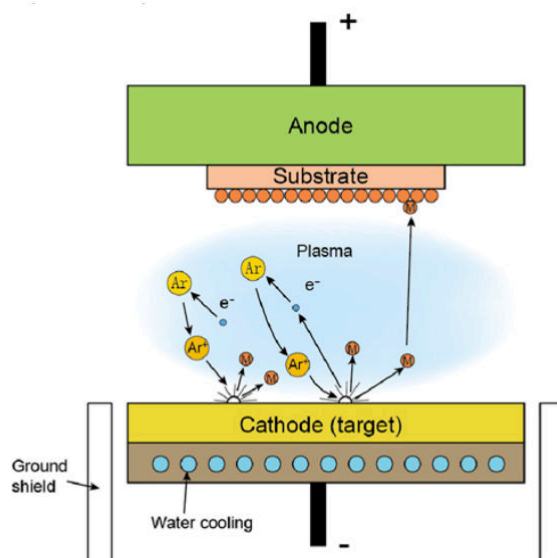


Figure 1.7. Basic components of DC diode sputtering system. Ionized by strong potential difference, argon atoms are accelerated towards the target. By this bombardment target atoms are ejected and eventually form a coating on the substrate [107].

Pulsed laser deposition. This deposition technique is extensively used to produce and investigate technically important and novel surfaces.

Typical PLD process undergoes in a vacuum chamber and consists in the laser ablation of target material that is placed in front of the substrate. High energy pulsed laser beam is focused on the target through the system of lenses and when it reaches the target, there is local rapid heating of the surface that results in target vaporization and formation of

a so-called plume. Thus created plasma plume contains ions, electrons, atoms, molecules, clusters and even melted macroparticles of a target material. These species then propagate towards the substrate where the coating is deposited [113]. Laser sources that are commonly used to ablate the target are excimer gas lasers KrF (248 nm), ArF (157 nm), XeCl (308 nm) and solid state laser Nd: YAG (255-1064 nm) [114–116].

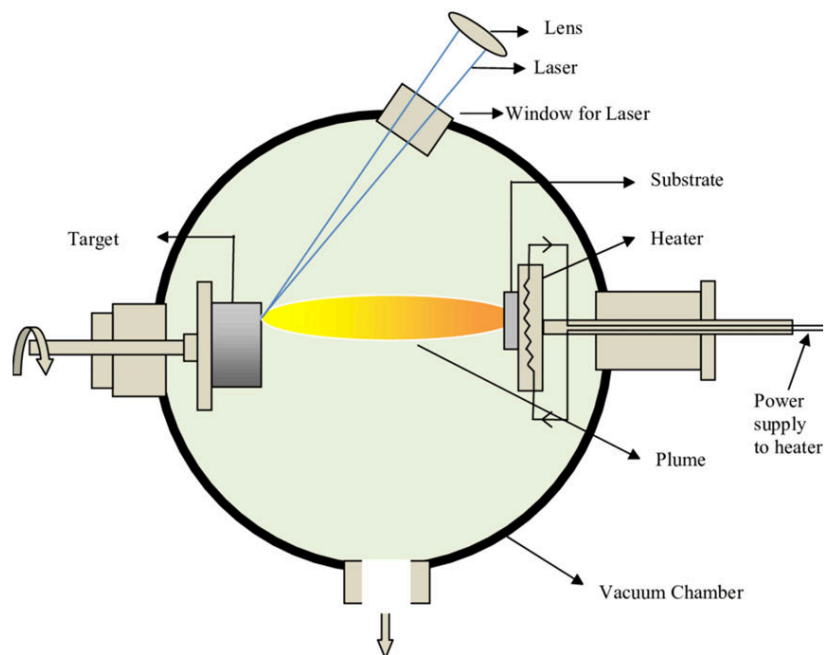


Figure 1.8. Schematic of PLD system. In this process films are grown as a result of thermal evaporation of the target material by laser ablation [112].

Although this method is not suitable for production of the samples with large surface area, it has growing interest, as laser deposition provides one of the fastest ways to synthesize a high quality coating with an excellent adherence to the substrate. Moreover, compared to conventional methods of thin films synthesis, PLD method leads to a better control of stoichiometry and crystallinity of the coating. Thin film quality and properties can be tuned with changing various parameters of the deposition, such as laser wavelength and energy density, laser repetition rate, distance between substrate and the target, temperature regime and gas pressure inside the vacuum chamber. Furthermore, the thickness of the coating can be very well controlled by choosing the appropriate number of laser pulses and different

gases (O_2 , N_2 , H_2 , Ar and others) can be introduced in the chamber during the deposition. The PLD system can be also equipped with rotational multi-target holder together with the mobile set of masks, thus allowing creation of combinatorial libraries of various thin film compositions on the surface of a single substrate, as well as deposition of multilayered coatings. Another advantage of PLD system is the possibility of utilizing setup features that bring opportunity to improve control of the effect of deposition conditions on material quality and properties. For example, reflection high-energy electron diffraction (RHEED) facility can be installed [117]. This technique allows to monitor growth of thin films in situ by analyzing the number and structure of deposited layers.

Hence, PLD provides a versatile and powerful tool for synthesis of thin films for wide range of applications. It is being applied to grow superconductive coatings, complex oxide systems and other functional materials [118–120]. Concerning studies of biomaterials, it was successfully used to deposit biocompatible coatings including hydroxyapatite (HA), which are of great interest in the studies of bone regeneration thanks to their resemblance to natural bones [121, 122]. For example, Rajesh et al. in [122] used PLD technique to deposit stable and adherent hydroxyapatite layer over titanium substrate with an interlayer of titania.

Outline

To conclude, the aim of this thesis is to show the feasibility of using PLD-deposited underused metal oxide thin films to evaluate human bone-marrow derived MCSs and cancer cells behavior in their contact with oxides. The PLD system for thin film synthesis allows to introduce O_2 flow into the chamber during deposition process, as well as to change deposition parameters such as temperature, pressure inside vacuum chamber and number and energy of applied pulses. This leads to the opportunity to study the effect of not only the chemical composition of ablated oxide target, but also the effect of all these deposition parameters on the mesenchymal stem cells and cancer cells behavior.

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2 Methodology

2.1 Preparation of oxide thin films via Pulsed laser deposition

Thin films were deposited on glass substrates using the PLD technique. TiO_2 , Al_2O_3 and VO_x targets were prepared by a standard solid state route. Microscope glass round cover slips were used as substrates ($\varnothing = 12$ mm). They were glued with silver paste on the heater with circular mask, this way, it was possible to avoid contamination of the substrate by Ag paste.

The deposition was performed using KrF excimer laser source ($\lambda = 248$ nm). For each sample the number of pulses was 5000 with frequency repetition rate of 5 Hz. The deposition was carried out at different temperatures in the range of 100 - 500°C. Prior to every deposition, the vacuum chamber was evacuated down with a turbo pump to a base pressure lower than 1.33×10^{-4} Pa (10^{-6} Torr). During experiments involving deposition with O_2 , gas was introduced in the chamber with a flow rate that led to the pressure 10^{-4} - 10^{-5} Torr.

2.2 Characterization of deposited thin films

2.2.1 X-ray diffraction

X-ray diffraction (XRD) is one of the most used methods of analysis in the material science. Diffraction peaks result from the interference of X-ray waves scattered by a set of parallel planes. The position of these peaks is defined by the distance between such planes of atoms. In order for diffraction to occur and for the scattered by a system of parallel crystal planes X-rays to give a maximum of intensity, it is necessary to fulfil the following condition (Bragg's law) [1]:

$$2d\sin\Theta = n\lambda,$$

where d is the interplanar distance, Θ is the scattering angle, n is any positive integer and λ is the wavelength of incident X-rays.

Within this study, XRD patterns were recorded using BrukerTM D8 Discover diffractometer with $\text{CuK}\alpha_1$ radiation ($\lambda = 1.541 \text{ \AA}$) and 2Θ angle range from 10 to 50° .

2.2.2 X-ray reflectivity

The thickness of the samples was characterized using X-Ray reflectivity (XRR) measurements, where the relative position of the oscillation maxima vs the square of their order number was fitted to obtain the film thickness from the slope. Such oscillations appear as a result of interference between the X-rays scattered from the surface of the thin film and the interface between substrate and thin film. Reflectivity curves were recorded using BrukerTM D8 Discover diffractometer with $\text{CuK}\alpha_1$ radiation ($\lambda = 1.541 \text{ \AA}$) and 2Θ angle range from 0 to 6° .

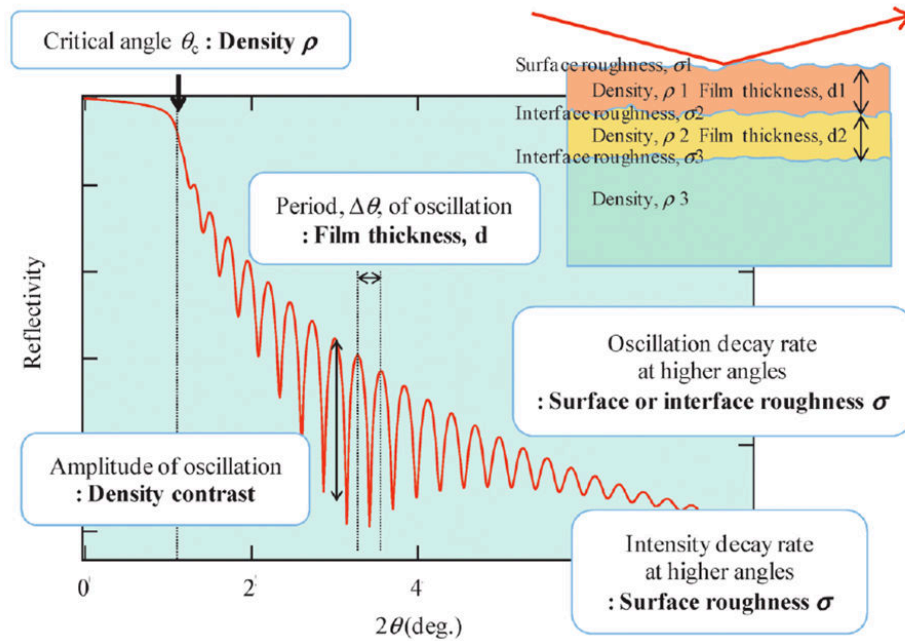


Figure 2.1. Information provided by X-ray reflectivity profile [2].

It is worth to note that XRR can be used to obtain other type of information, such as density and surface or interface roughness (Fig 2.1).

2.2.3 Atomic force microscopy

Surface morphology study was done by Atomic Force Microscopy (AFM, PicoSPMTM) technique. The operating principle of AFM is based on the effect of forces from the sample surface on the tip of scanning cantilever (Fig. 2.2).

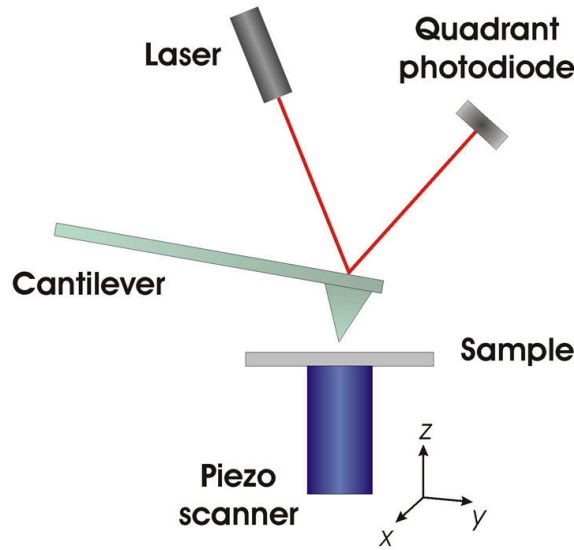


Figure 2.2. 2D schematic of AFM working principle. Adapted from [3].

The force acting on the probe from the surface leads to bending of the console. By registering the degree of bending, it is possible to control the force of interaction of the probe with the surface. Depending on the nature of the force acting between the cantilever and the sample surface, there are three modes of operation of an atomic force microscope: 1) contact, 2) non-contact, 3) tapping modes. In the contact mode the probe tip directly touches the sample surface during scanning. In the non-contact mode the probe is far enough and do not touch the surface. In the tapping mode the intermittent contact occurs. The last two AFM modes are needed to implement modulation (or vibration) techniques. Each mode is used to solve particular problems, some investigations being conducted using

various techniques in different modes. This gives the researcher a wide scope of opportunities and allows operating a microscope in a most appropriate and effective mode under given experimental conditions.

For the current study, the root-mean square (rms) roughness was derived from AFM measurements of $1\ \mu\text{m}^2$ area in the tapping mode, and calculated with WSxM 5.0 software.

2.2.4 Water contact angle measurements

One of the important characteristics of material surface is its wettability. Wettability is determined by the value of the contact angle. The contact angle is formed by the flat surface of the solid and by the plane tangent to the free surface of the liquid passing through the point of the interception of the solid, liquid and gas phases. Depending on the value of the contact angle, surface is considered to be hydrophilic with $\Theta < 90^\circ$ or hydrophobic with $\Theta > 90^\circ$ (Fig. 2.3). Wettability is a surface phenomenon involving the interaction of a liquid with the surface of a solid or other liquid. There are two types of the wettability: immersion and contact. The value of wettability depends on the ratio between the forces of adhesion of a liquid molecule with molecules of a wetted body and the forces of mutual adhesion of molecules of a liquid.

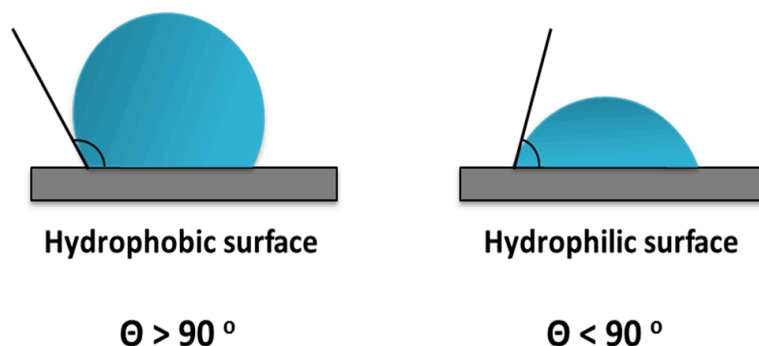


Figure 2.3. Contact angle schematic for hydrophobic and hydrophilic surfaces.

The contact angle of surfaces was evaluated by a drop shape analysis system (DSA, Kruss, Germany) by the sessile drop technique ($1\ \mu\text{l}$ droplet). For each sample, final mean contact angle was obtained after at least three measurements.

2.3 Cell culture

2.3.1 Human bone-marrow derived mesenchymal stem cells

Bone marrow was obtained from iliac crests of adult donors undergoing arthroplasty that signed an agreement form according to local ethical committee. Marrow was aspirated and cells were fractionated on a Hypaque-Ficoll density gradient. Mononuclear cells were isolated and expanded in alpha-MEM medium supplemented with 10% fetal calf serum (InvitrogenTM, Cergy-Pontoise, France), 2 mM L-glutamine, 1 ng/ml FGF-2 (SigmaTM), and antibiotics. Close to 80% of confluency, the cells were harvested by trypsinization (0.25% trypsin/1 mM EDTA, InvitrogenTM) and seeded at 2×10^3 - 5×10^3 cells/cm². After 5 passages, cells were checked for hematopoietic markers absence by RT-PCR. For *in vitro* experiments hBMMSCs were seeded at the density of 5×10^4 cells/cm² in the 24-well plate (Fig. 2.4).

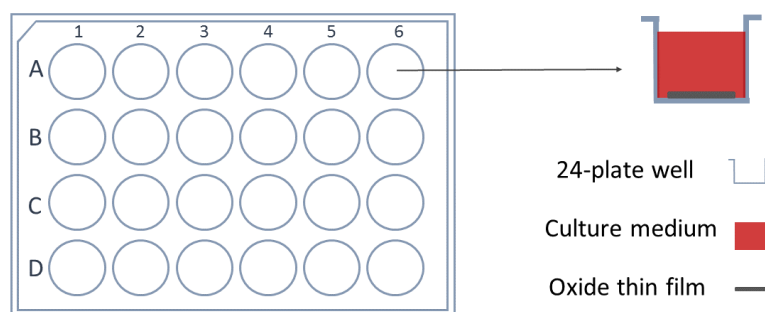


Figure 2.4. Schematic of 24-well plate used during in-vitro experiments.

Culture medium was changed 3 times per week and kept in a 5% CO₂ atmosphere at 37°C.

2.3.2 Ovarian cancer cells

The human chemoresistant ovarian carcinoma cell lines SKOV3 and IGROV1-R10 were grown in RPMI 1640 medium supplemented with 2 mM GlutamaxTM (Fisher Scientific Bioblock, Illkirch, France), 10% fetal calf serum (Fisher Scientific Bioblock), 20 mM HEPES and 33 mM sodium bicarbonate (Gibco BRL, Lyon, France) and were maintained in a 5% CO₂ humidified atmosphere at 37°C.

For kinetic measurement of cytotoxicity IGROV1-R10 cells were seeded at 55×10^3 cells per well in 24-well plates in RPMI-1640 media, while SKOV3 cells were seeded at 30×10^3 cells per well. Cells were cultured at 37°C and 5% CO₂ and monitored using an IncuCyte S3 (Essen BioScience). CellToxTM Green Cytotoxicity Assay reagent (Promega) was added and baseline images were taken using 10x objective. The plate was scanned and fluorescent and phase-contrast images were acquired in real time every 1 hour from 9 separate regions per well. The CellToxTM Green reagent labels dead cells yielding green fluorescence. The live-cell phase contrast images were used to calculate confluence using the IncuCyte software, and to provide morphology information. Each experiment was done in triplicate and accumulation of CellToxTM Green over time was normalized to confluence of cells.

2.3.3 Chondrosarcoma cells

The human chondrosarcoma cell lines SW1353, FS090, CH2879 and JJ012 were seeded at 10^4 cells per well in 24-well plates in DMEM medium supplemented with 2 mM GlutamaxTM (Fisher Scientific Bioblock, Illkirch, France), 10% fetal calf serum (Fisher Scientific Bioblock), 20 mM HEPES and 33 mM sodium bicarbonate (Gibco BRL, Lyon, France) and were maintained in a 5% CO₂ humidified atmosphere at 37°C.

2.4 Study of cell adhesion and proliferation

2.4.1 Phase contrast microscopy

Phase contrast microscopy allows to get clear images of transparent objects that are usually hard to observe in a bright field. With this method it is also possible to study living cells and tissues in dynamics. The main principle of phase contrast microscopy is conversion of electromagnetic wave phase shift into a contrast of light intensity distribution in the image [4].

Cell adhesion and proliferation were monitored by means of phase contrast VisiScopeTM series 400 microscope (VWR) and EVOSTM FL Auto 2 Imaging system (Thermo Fisher Scientific). To follow multicellular structures diameter evaluation, hBMMSCs were seeded at the density of 10^4 cells/cm² in a 24-well plate containing thin films of TiO₂ and Al₂O₃ or glass substrate as control. Media were changed 3 times per week, and kept in a 5% CO₂ atmosphere at 37°C during 26 days. For each sample, multicellular structures were observed under phase contrast VisiScopeTM series 400 microscope (VWR).

2.4.2 WST-1 Cell viability and proliferation assay

WST-1 is a colorimetric assay that is applied for evaluation of cytotoxicity, cell viability and proliferation [5]. WSTs (water-soluble tetrazolium salts) are family of dyes and main principle of WST-1 assay is based on detection of viable cells when this dye is reduced outside the cell. Reduction leads to a subsequent change of color, which was detected by optical density measurements with Multiskan GO spectrophotometer (Thermo Scientific). Absorbance was registered at wavelength equal to 450 and 600 nm. This test was used solely to compare cytotoxicity of VO_x thin films deposited under normal and oxygen partial pressures with glass control.

2.4.3 Histological staining

For histological staining hBMMSCs were seeded at the density of 10^4 cells/cm² in a 24-well plate on different substrates including uncoated glass slides, as well as slides with oxide thin films. Media were changed 3 times per week, and kept in a 5% CO₂ atmosphere at 37°C during 14 days. After, cells were rinsed twice with PBS and fixed with 4% paraformaldehyde for 15 minutes prior to another rinse with PBS, then cells were kept in this buffer until further analysis. For each sample, the results of Van Gieson, Hematoxylin phloxine saffron and Masson's Trichrome staining were fixed on microscopic histological glass slides with solution of an acrylic polymer and observed with EVOSTM FL Auto 2 Imaging system (Thermo Fisher Scientific).

Van Gieson staining. First, glass slides were placed twice in a row into xylene for 3 minutes. Second, slides were dipped 20 times (twice) into 99% ethanol and after in Weigert's iron hematoxylin staining solution for 10 minutes. Afterwards, slides were rinsed with tap water, left for two minutes in picrofushin (Van Gieson) solution and dipped into distilled water 10 times. Then, samples were rinsed in two changes of 99% ethanol and dipped 20 times with changes in xylene.

Hematoxylin phloxine staining. Slides with cells kept in xylene for 3 minutes two times, dipped into two changes of 99% alcohol 10 times and rinsed with tap water after. Then, they are placed into Harris hematoxylin for 3 minutes and again rinsed with tap water. After 3 dips in acid alcohol in order to remove excessive amounts of stain, glass slides were dipped 10 times into tap water and 1% lithium carbonate. Then, after washing again with tap water, samples were kept for 5 minutes in 2% phloxine, rinsed with water and dehydrated with 3 changes of 99% alcohol. Same dehydration procedure was made after keeping slides on saffron staining solution for 2 minutes. Finally, 10 dips in the xylene were made twice.

Masson's Trichrome staining. Glass slides were left twice in xylene for 3 minutes and dipped in 99% ethanol 2×20 times. Then, they were kept in Weigert staining solution for 3 minutes and rinsed well with tap water before dipping them in Ponceau-Fuchsin (Masson)

solution for 5 minutes. Next step was to wash slides with 1% acetic acid prior to their treating by phosphomolybdic acid for 5 minutes. Slides were rinsed again with acetic acid solution and placed for 1 minute in analine blue. Finally, after another rinsing with acetic acid, slides were dehydrated with 99% ethanol (2×10 dips) and cleared in xylene (2×10 dips).

2.4.4 Scanning electron microscopy

Among the techniques that are widely used for surface characterization, scanning electron microscopy is one of the most common. In this type of microscopy, the image is formed by various signals generated sequentially as a focused electron beam scans the surface of the sample with a system of deflection coils. The beam of electrons with energies ranging from a few hundred eV to 50 keV is focused on the surface of the sample into a very small spot of about 5 nm in diameter. Signals are mainly coming from flow of electrons produced after interaction with a material. Depending on the type of electrons, i.e. backscattered, secondary and others, different information can be obtained. SEM method allows both qualitative and quantitative analysis, with simultaneous examination of the size and shape of grains, phase and grain and size distribution, determine the phase composition and distribution of chemical elements by its area.

Morphology of MSCs cultured onto oxide thin films was studied with Scanning Electron Microscope (SEM) ZeissTM SUPRA 55 (Fig. 2.5), operated at the range of voltages 2.0 - 10.0 kV, and 4 - 10 mm distance.

In our study, hBMMSCs cultured during 2 weeks were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH = 7.4) at 4°C, rinsed with cacodylate buffer and postfixed in 1% osmium tetroxide. Then, they were dehydrated progressively with ethanol (70 - 100%) and dried in a critical point dryer (CPD 030 LEICA Microsystem). Samples were sputtered with platinum (Pt) and observed with a SUPRA 55 scanning electron microscope (ZeissTM).



Figure 2.5. Zeiss SUPRA 55 scanning electron microscope.

2.4.5 Cell density measurements

For mesenchymal stem cells population growth measurements, HBMMSCs were seeded at the density of 10^4 cells/cm² in a 24-well plate containing thin films of TiO₂ and Al₂O₃, or glass substrate as control. Media were changed daily for fresh medium during five days. Cells were harvested and numbered separately using a Malassez counting chamber. The doubling time T_d is calculated from the cell growth curve over 5 days using the following equation: $T_d = (T_f - T_i) \times \log 2 / (\log N_f - \log N_i)$,

where T_f , T_i , N_f and N_i represents the final time, the initial time, the final cell number, and the initial cell number, respectively.

Later in the study, for the experiments concerning using same deposition temperature for oxide thin films, hBMMSCs were seeded with α -MEM medium at the density of 104 cells/cm² in a 24-well plate onto different substrates, including thin films of TiO₂, VO_x and Al₂O₃ or uncoated glass slide. After two hours, the media were removed and the

cells were washed with phosphate-buffered saline to remove non-adherent cells. hBMMSCs were then harvested by trypsinization (0.25% trypsin/1mM EDTA, InvitrogenTM) and numbered separately using a trypan blue staining and CountessTM II Automated Cell Counter (Thermo Fisher Scientific). Same procedure was carried out to count cells after culturing at different times (2, 4 and 7 days).

2.5 Study of human mesenchymal stem cell differentiation

2.5.1 Differentiation staining

In order to assess the influence of oxide thin films on the ability of MSCs to differentiate into various type of cells, differentiation staining was performed. For this, MSCs were cultured with standard α -MEM media (without basic fibroblast growth factor) during 7 days. Then, the chondrogenic, osteogenic and adipogenic differentiation media were added for alcian blue, alizarin red and oil red stainings, respectively. The media were changed each 2 - 3 days in both cases. After 14 days of culture in total, cells were fixed with 4% paraformaldehyde (PFA) and after being washed with phosphate-buffered saline (PBS), cells were kept in 1.5 ml of PBS for further differentiation staining tests. To fix samples after staining on microscopic histological glass slides, solution of an acrylic polymer was used.

Alcian blue staining. To identify chondrogenic differentiation, MSCs were stained for acid mucopolysaccharides, which are markers of chondrogenesis. Mucopolysaccharides(or glycosaminoglycans) are linear complex polysaccharides that have structural function in connective tissues [6]. Alcian blue staining was used to highlight specifically sulfate and carboxylic mucopolysaccharides. 1% Alcian blue solution (pH = 2.5) was prepared by dissolving 1 g of Alcian blue powder in 100 ml of 3% acetic acid. After aspiration of PFA, cells were washed with distilled water (H₂O_d) and stained with alcian blue solution for 30 minutes.

Then, cells were washed 4 times with H₂O and samples were glued on microscopic glass slides. For optical density analysis, samples were washed with 1 ml of H₂O four times and after 1% sodium dodecyl sulphate (SDS) was added and obtained solutions were transferred to the 48-well plate. To analyze the cells, the absorbance at 616 nm was determined for triplicate samples (Multiskan GO spectrophotometer, Thermo Scientific) .

Alizarin red staining. This staining is used to detect osteogenic differentiation of cells [7]. Undifferentiated MSCs (without extracellular calcium deposits) should appear slightly reddish, whereas MSC-derived osteoblasts (with extracellular calcium deposits) are supposed to be orange-red. Alizarin red solution was prepared by dissolving 2 g of Alizarin red S in 50 ml of distilled water. Then, pH was adjusted to 4.1 - 4.3 by adding HCl or NH₄OH. To stain the cells, H₂O was carefully aspirated after washing and Alizarin red staining solution was added to cover cellular monolayer. Incubation was carried out during 30 minutes in the dark at room temperature. Afterwards, staining solution was aspirated, cells were washed 4 times with H₂O and samples were glued on microscopic glass slides. For optical density tests, cells were washed four times with 1 ml of H₂O and after 1 ml of H₂O was added. The absorbance at $\lambda = 490$ nm was determined for triplicate samples.

Oil red staining. To highlight lipids and evaluate the adipogenic differentiation Oil red staining is commonly used [8]. First, stock solution of Oil red was prepared by adding of 0.3 g of Oil red O powder to 100 ml of 99% isopropanol. Second, three parts of stock solution were mixed with two parts of distilled water and filtered with Whatman filter paper in order to prepare working solution. Then, H₂O was aspirated after washing, 2 ml of 60 % isopropanol were added to each well and left for 5 minutes. Finally, isopropanol was removed and cells were stained by adding 2 ml of Oil red working solution to each well. Stained cells were incubated for 30 minutes at room temperature in the absence of light. After that, staining solution was carefully aspirated and cells were washed four times with 1 ml of H₂O and samples were glued on microscopic glass slides for observation.

2.5.2 Real time RT-PCR

Real-time reverse transcription polymerase chain reaction (RT-PCR) is widely used method for measuring gene expression (Fig. 2.6).

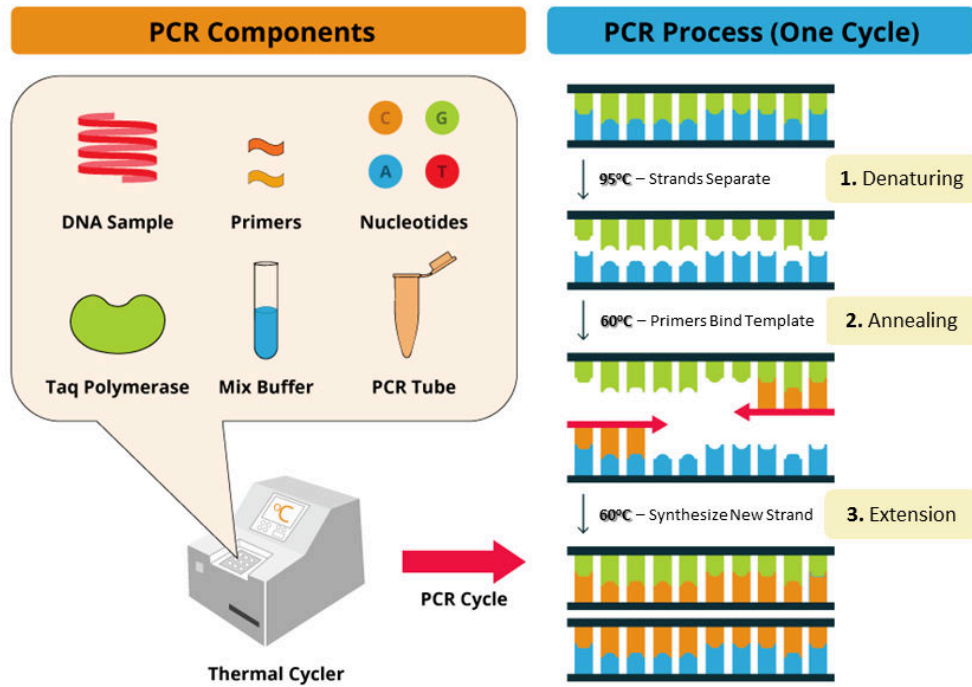


Figure 2.6. Schematic of PCR process (adapted from [9]).

This technique is based on the amplification of sequences of complementary DNA (cDNA) and further detection of amplified products. When desired template DNA is synthesized, it is doubled in the amount by repeated cycles of heat denaturation until it reaches reaction plateau. Then, by means of fluorescence, the amount of product is measured, where its sufficient amount significantly increases the signal fluorescence. Higher amount of copies in the beginning of the process leads to fewer amplification cycles. Cell culture was done with using standard (α -MEM) and differentiation media. COL2, ACAN, COMP, SOX9, ELN and others were tested for chondrogenesis, i.e. cartilage formation. OMD, RUNX2 and SPARC were tested for osteogenesis, i.e bone formation.

RNA extraction. RNeasy mini kit (Qiagen) was used for purification of total RNA extracted from the cells. Buffer RLT was used for cell lysis and homogenization. When the cells were harvested, 700 μl of the sample was transferred to RNeasy Mini spin column placed in a 2 ml collection tube and centrifuged for 15 seconds at $\geq 8000 \times g$. Then, 700 μl buffer RW1 was added to spin column and and centrifuge for 15 seconds at $\geq 8000 \times g$. Flow-through was discarded. Next step was repeated twice and consisted in adding 500 μl of buffer RPE to the RNeasy spin column and centrifuging for 15 s the first time and for 2 minutes a second time. RNeasy spin column was placed in a new 1.5 ml collection tube, 30 μl RNase-free water were added directly to the spin column membrane and the sample was centrifuged for 1 minute at $\geq 8000 \times g$ to elute the RNA.

Quantification of RNA. Multiskan GO spectrophotometer (Thermo Scientific) was used to assess RNA concentration by measuring absorbance at $\lambda = 260 \text{ nm}$. Depending on the obtained values for amount of RNA for each sample from various conducted experiments, the concentration used for further steps was calculated according to the available sample volume and was equal to 100, 500 or 1000 ng/ μl .

DNase treatment and its validation. Purpose of DNase treatment step is to remove traces of contaminating genomic DNA (gDNA) in the RNA material extracted from the samples. In order to do that, H_2O (DEPC) was mixed with appropriate volume of the RNA samples, then, 10x reaction buffer and DNase I, Amplification Grade, were added for the total volume of the mix $V_{\text{total}} = 15 \mu\text{l}$. This final mix was gently mixed and incubated for 15 minutes at room temperature. After, 1 μl of Stop Solution was added to bind calcium and magnesium ions and to inactivate the DNase I. After, the mix was heated at 70° for 10 minutes. In order to ensure that all traces of gDNA was removed by treatment with DNase, a PCR reaction was performed with b-actin primers before and after DNase treatment, then, samples were electrophoresed on a 1% agarose gel stained with GelRed.

Reverse transcription (RT). During this step, complementary DNA (cDNA) is synthesised from RNA template. This process employs reverse transcriptase, an enzyme, which plays the role of a catalyst. According to the kit's instruction 10 μl of the DNase-treated samples

were added to 10 μl of the mix consisting of DEPC H_2O , 5x buffer first strand, Oligo dt, dNTPs and enzyme M-MLV RT (Nucleospin, Macherey Nagel). The total volume V_{total} was equal to 20 μl . Then, samples underwent temperature treatment in Bio-Rad thermal cycler (Applied BiosystemsTM) with the following steps: 50 minutes at 37°C, 15 minutes at 70°C (deactivation of enzyme) and cooling down to 12°C.

Real-time PCR. There are three main stages during PCR process, namely:

- Denaturation. During this step the DNA double strands are separated by heating at temperatures higher than 90°C.
- Annealing. When the temperature is decreased and is equal to the value that is a little below melting temperature value, reverse and forward primers bind to the strands of DNA template.
- Elongation. During third stage of PCR, DNA polymerase is enabled to add nucleotides to the annealed primer.

OMD, RUNX2, ELN and SPARC genes were tested after cell culture in osteogenic medium. COL2, ACAN, COL1, COMP, ELN and SOX9 were tested after cell culture in chondrogenic medium. Expression of PPARG was analyzed with regards to adipogenic differentiation. The sequences of these primers and housekeeping genes used during these experiments, i.e. GAPDH, RPL13, B2MG and actin can be seen in Table 2.1. SYBR Green dye was used for the detection of double-stranded DNA and mRNA expression levels were calculated by $2^{-\Delta\Delta C_t}$ method.

Table 2.1

Primer sequences used for PCR analysis

Gene	Primer	Gene	Primer
COL1 (collagen type I alpha 1)	F: CACCAATCACCTGCGTACAGAAC R: CAGATCACGTCATCGCACAAAC	RUNX2 (runt-related transcription factor 2)	F: GTGATAAATTCAGAAGGGAGG R: CTTTGTCTAATGCTTCGTGT
OMD (osteomodulin)	F: AGGCTGTGTCAGTGAATGCTT R: TTGGATAGTCTTGAGTTTGCAT	ELN (elastin)	F: CCAGGTGTAGGTGGAGCTTT R: CCATAGCCATAGGGCAGTTT
SPARC (secreted protein acidic and cysteine-rich)	F: CCCATTGGCGAGTTTGAGAAG R: CAAGGCCCGATGTAGTCCA	PPARG (Peroxisome proliferator-activated receptor gamma)	F: GGCTTCATGACAAGGGAGTTTG R: ACTCAAACCTGGGCTCCATAAAG
COL2 (collagen type II)	F: TGGACGATCAGGCGAAACC R: GCTGCGGATGCTCTCAATCT	Actin (beta-actin)	F: AGACCTGTACGCCAACACAG R: CGATCCACACGGAGTACTTG
ACAN (aggrecan)	F: GTGCCTATCAGGACAAGGTCT R: GATGCCTTTCACCACGACTTC	GAPDH (glyceraldehyde-3-Phosphate Dehydrogenase)	F: ATGGGGAAGGTGAAGGTCG R: TAAAAGCAGCCCTGGTGACC
COMP (cartilage Oligomeric Matrix Protein)	F: GCTCAGGGCCCTCATAGAT R: TGGGTTGGAAGGACAAGAAG	RPL13 (ribosomal protein L13)	F: GTTCGGTACCACACGAAGGT R: CTGGGGAAGAGGATGAGTTTG
SOX9 (SRY (sex determining region Y) – Box 9)	F: CCCATGTGGAAGGCAGATG R: TTCTGAGAGGCACAGGTGACA	B2MG (beta-2-microglobulin)	F: GAGGCTATCCAGCGTACTCCA R: CGGCAGGCATACTCATCTTTT

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3 Results and Discussion

3.1 Characterization of deposited thin films

As it was mentioned in the first chapter, interaction between material surface and biological matter, e.g. cells, is highly dependent on a number of factors, such as surface chemical composition, its morphology and porosity, crystallographic structure, wettability and others. The cellular response and behavior while in contact with material can be very sensitive to even small change in its physical or chemical properties. Thus, it is important to pay due attention to the appropriate characterization of the material before utilizing it for cell cultures and biological tests. Within this study, prior to *in vitro* experiments, various techniques were used in order to obtain data on film thickness, roughness, wettability and some other properties. More than 350 samples have been utilized for cell culture and results of analysis are proving good control and reproducibility of pulsed laser deposited thin films' properties.

3.1.1 X-ray diffraction

Oxides of titanium and aluminum can undergo temperature- or pressure-induced phase transitions [1, 2]. However, despite all possible phases, in regards to oxidation state of the metals, they are mostly stable with the following metal-oxygen ratio: TiO_2 and Al_2O_3 (often referred as titania and alumina). Namely with such chemical formulas they exist in nature as minerals, where rutile and corundum are most abundant for TiO_2 and Al_2O_3 , respectively.

Unlike titanium and alumina, vanadium forms various oxides depending on the temperature and other conditions, this can be seen from phase diagram represented in Fig. 3.1a [3]. In order to get the information which vanadium oxide specifically was deposited during our experiment, X-ray diffraction was carried out. XRD measurements revealed that deposited thin films have amorphous structure. Diffraction patterns for vanadium oxide thin films deposited on glass substrates at 300 and 500°C presented in Fig. 3.1b, where two broad peaks can be seen. Such peaks with low intensity appear in case of the lack of long range order

in the structure. XRD data indicate that elevated temperatures can lead to the changes in crystallographic structure as it can be seen that vanadium oxide deposited at 300°C exhibit more amorphous structure as the diffraction peaks appear broader and with lower intensity in comparison to the film deposited at higher temperature. Nevertheless, X-ray diffraction analysis did not allow to have certainty about particular stoichiometry of vanadium oxide and, therefore, hereinafter composition of vanadium oxide thin films will be referred as VO_x .

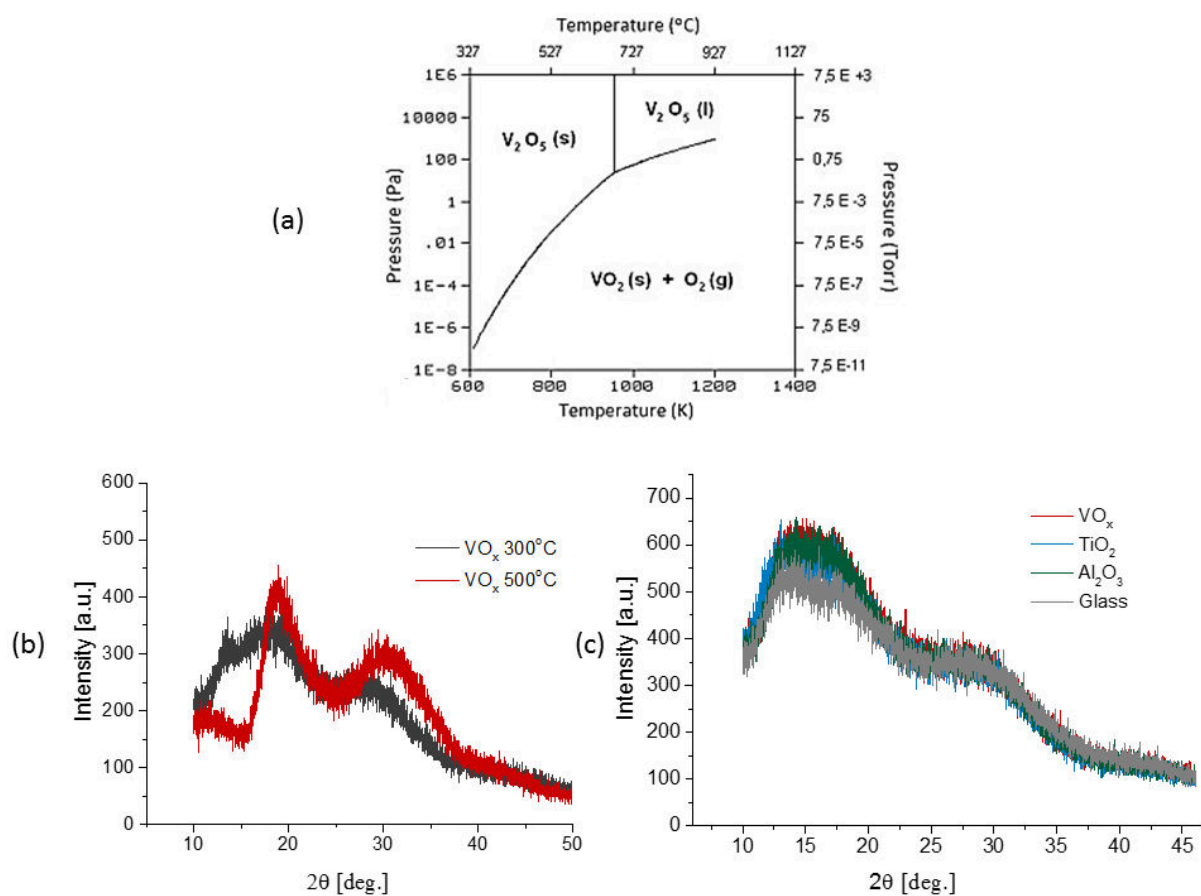


Figure 3.1. Temperature-pressure diagram of the vanadium-oxygen system (a). XRD patterns of VO_x thin films deposited at 300 and 500°C. (b). Comparison of XRD patterns of VO_x , TiO_2 and Al_2O_3 thin films deposited at 300°C and glass substrate heated up to the same temperature (c).

Additionally, XRD measurements were made for three oxides deposited at 300°C and glass substrate heated up to the same temperature (Fig. 3.1c). Comparison of this data

leads to a conclusion that observed peaks come mostly from glass substrate and it is not possible to distinguish peaks related to the oxides in this case.

Amorphous structure of thin films is related to the amorphous nature of the glass substrate and to the fact that all films were deposited at significantly lower temperature than the temperature of their crystallization. This property, i.e. crystallographic structure, should be taken into account as the parameter that can have an influence on cellular behavior. For example, Lv et al. in [4] observed enhanced osteogenic response of mouse pre-osteoblast (MC3T3-E1) cells on the anatase TiO_2 film in comparison with rutile TiO_2 film. This effect was associated with larger amount of hydroxyl groups on the surface of anatase that induced the change in the conformation of the adsorbed fibronectin, which led to the exposure of more cell-binding sites. Cervantes et al. also demonstrated how viability and proliferation of Chinese hamster ovary (CHO-K1) cells can be affected by the ratio of anatase to rutile on the TiO_2 thin films [5].

There are a few ways to get a crystallized thin film. One of the approaches to obtain thin film with ordered structure is annealing, which often is done after deposition [6]. Crystallization process occurring at the very high temperatures is explained by the increased rate of atom movements, leading to partially or completely elimination of structural heterogeneity. Another way to get a crystalline thin film is to use single crystal substrate like silicon, SrTiO_3 and others. In this case, highly ordered structure can be formed as a result of epitaxial growth which implies oriented growth of thin film [7, 8]. In the current study we used namely glass slides as substrates since they are transparent and, hence, it is possible to observe cells by the means of phase contrast microscopy.

3.1.2 X-ray reflectivity

Another important parameter that was measured for deposited coatings is the thickness and X-ray reflectivity (XRR), a surface-sensitive analytical technique, was utilized for this purpose. Data on thickness of films was calculated from reflectivity curves by using following

formula:

$$T = \sqrt{\lambda^2/4B},$$

where T is thickness of the film, λ is X-ray wavelength and B is a slope obtained from analyzing oscillation curves maxima. This calculation led to thickness values that fall within the range of 15 - 20 nm depending on the deposition conditions. Taking into account the number of applied pulses during each deposition, the deposition rate for these three oxides was estimated to be in the range of 0.03 - 0.04 Å/ pulse. Such values are relatively low, as comparing to, for example, sputtering techniques, and indicate that a precise control of the thickness is possible.

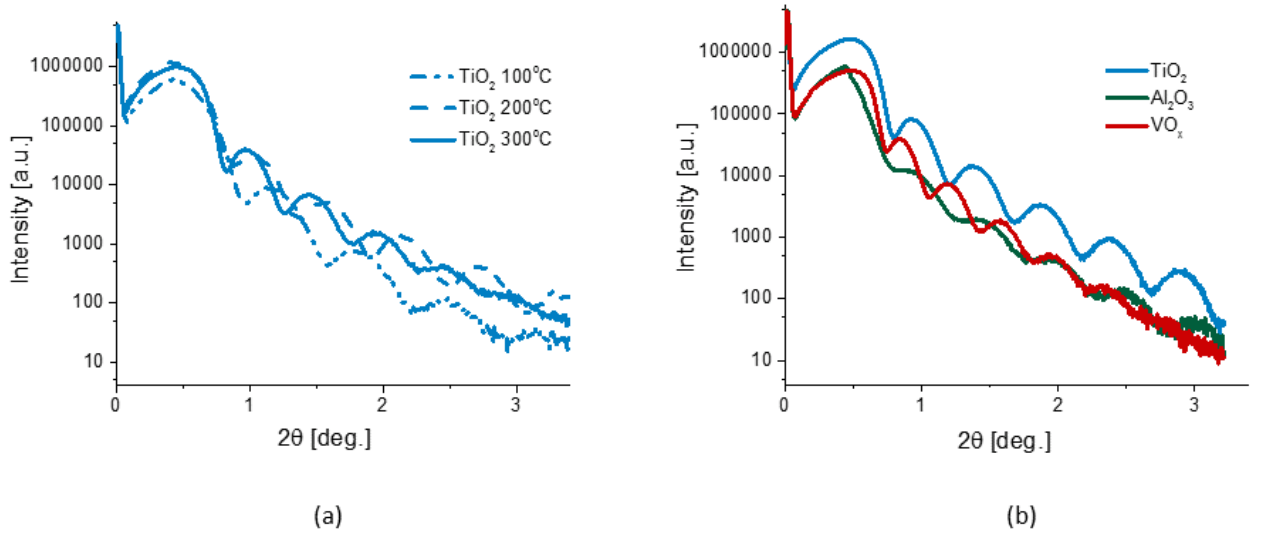


Figure 3.2. XRR curves of TiO₂ films deposited at various temperatures (a) and TiO₂, Al₂O₃, VO_x thin films deposited at 300°C (b).

Results of XRR analysis showed that depending on the target composition, thickness of oxide films deposited at the same temperature can vary (Fig. 3.2a). Since same laser energy and repetition rate were used during deposition of all three oxides, the variance in thickness

is associated with the difference in optical properties of the metal oxide solid targets. In turn, optical properties depend on the intrinsic electronic structure of particular metal oxide. Thus, it leads to different laser adsorption depth for each oxide and consequently ablation rate, which is the amount of material transferred from target to the substrate. In general, it can also be seen that an increase in the deposition temperature leads to the shorter periods of oscillations, i.e. higher values of the film thickness (Fig. 3.2b). This can be explained by the effect of the heating that causes an increase in mobility of ablated species.

3.1.3 Atomic force microscopy

In order to assess data on surface roughness, thin films of TiO_2 , Al_2O_3 and VO_x deposited at 100, 200 and 300°C were characterized by means of AFM technique. Obtained results indicated that temperature of the deposition had a little effect on surface roughness of films as explained below.

Comparison of surface profiles of glass substrate and oxide thin films deposited at the same temperature (300°C) also do not reveal big alteration in surface roughness (Fig. 3.3). It can be seen that despite the different composition, all deposited oxide films have uniform nanostructured surface with values of average roughness lower than 1 nm. Analysis of AFM measurements of thin films deposited within this study reveal that all the coatings have a very smooth and flat surface with the average grain size less than 100 nm. RMS values were found to be in the range of 0.1 - 0.5 nm, indicating that splashing is not significant.

As it was demonstrated in various studies, high surface roughness favors cell adhesion due to the fact that rough surface provides more additional sites for the adsorption of cell adhesion proteins. For example, Liao et al. [9] used AFM in order to see how UV irradiation and H_2SO_4 treatment affect surface roughness of TiO_2 films, which in turn could improve anticoagulant ability and endothelial cell compatibility of the films. Overall, any surface with appropriate values of roughness can have a lot of sites that naturally serve as anchor points for cells to attach. In the case of flat surfaces, the effect of surface roughness on

cellular behavior is minimized.

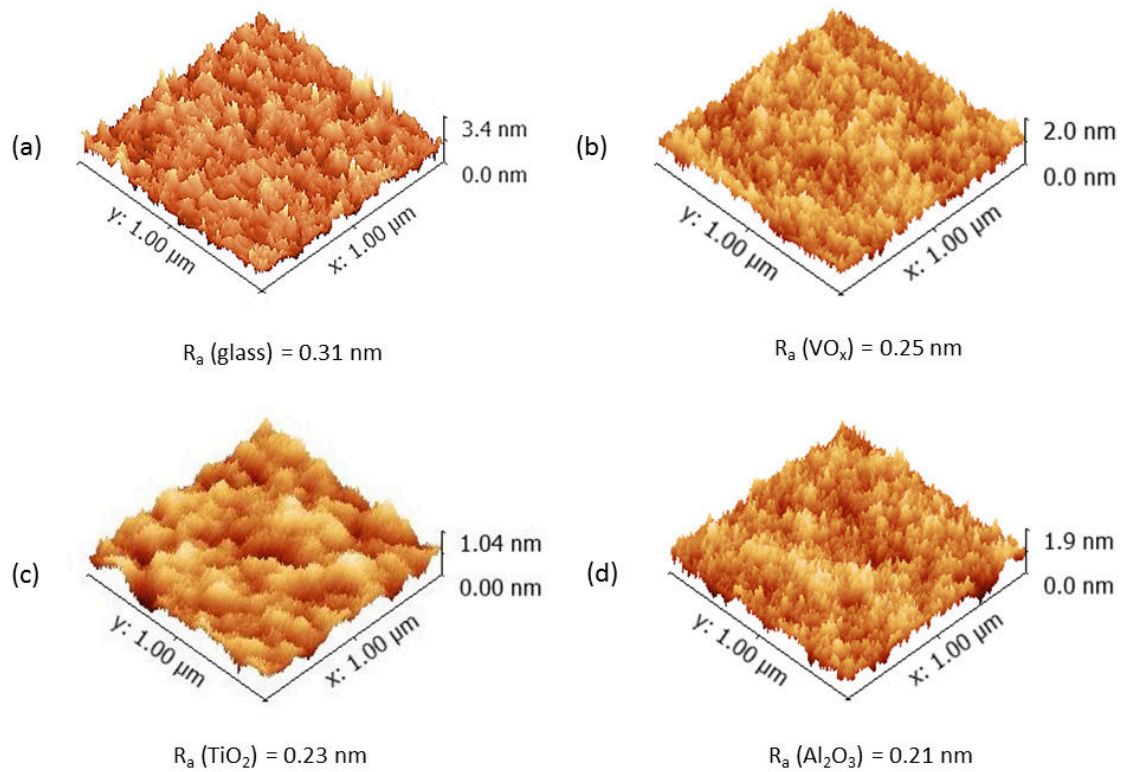


Figure 3.3. AFM images of surface profiles of glass substrate (a) and VO_x (b), TiO_2 (c), Al_2O_3 (d) thin films deposited at 300°C . Corresponding value of average surface roughness (R_a) is indicated below the surface profiles.

AFM analysis showed that PLD technique allowed to synthesize TiO_2 , Al_2O_3 and VO_x thin films with average surface roughness lower than 1 nm. Such values are significantly lower than those usually obtained with sputtering deposition techniques that are widely used. Moreover, regardless wide variety of deposition conditions, high level of results reproducibility for surface roughness was maintained, thus confirming that the laser ablation technique is appropriate and promising choice of method to deposit oxide coatings for studies on cellular behavior.

3.1.4 Water contact angle measurements

Subsequent to the roughness analysis with AFM, surface wettability properties were also investigated. Wettability is a solid-liquid-vapour interfacial phenomena that is characterized by measuring the water contact angle formed between a liquid drop and a solid surface. In this study, the contact angles were found to be equal to 61.72, 70.02 and 85.30° for VO_x , TiO_2 and Al_2O_3 films deposited at 300°C, respectively. This indicates that the aluminum oxide surface is less hydrophilic than surface of TiO_2 and VO_x . It is noteworthy that contact angle for non-treated glass substrate was higher than for glass heated up to 300°C, namely 44.31 vs 32.71° (Fig. 3.4a,b). The heating was carried out for about 30 minutes in order to reproduce the substrate heating during deposition. Taking into account results of XRD analysis, it can be seen that elevated temperatures lead to changes in the properties of the substrate.

Overall, in regards to oxide thin films, no significant difference was observed between the various growth conditions and surface of all analyzed films were hydrophilic with water contact angle less than 90 degrees (Fig. 3.4c-e).

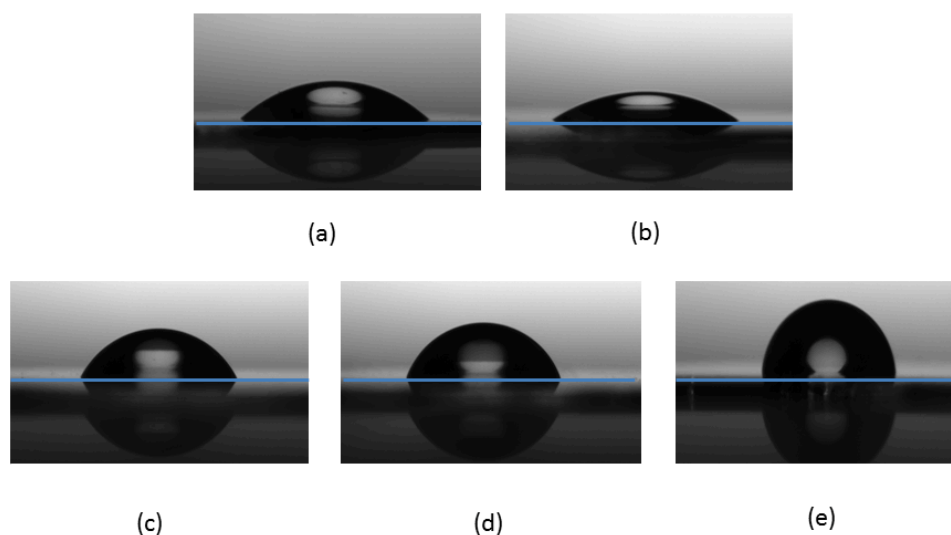


Figure 3.4. Water contact angle measurements for glass substrate (a), glass substrate held at 300°C for 30 minutes (b), and VO_x (c) TiO_2 (d), Al_2O_3 (e) thin films deposited at 300°C.

Hydrophilic nature of surface is known to have a positive effect on cell adhesion and spreading as the adsorption of extracellular matrix proteins is facilitated. However, this dependence is not always so straightforward. For example, Zhou et al. [10] cultured Tca-8113 epithelia-like cells on TiO_2 films, which were deposited by dc reactive magnetron sputtering on the glass substrates. After, cell behavior was studied and it was demonstrated that cells preferred to adhere and proliferate on the films with suitable values of roughness and wettability, i.e. neither on the most smooth and hydrophobic nor on the most rough and hydrophilic.

3.1.5 Energy dispersive X-ray spectrometry

Fig. 3.5 displays EDS spectra of TiO_2 and Al_2O_3 thin films deposited on glass slides. The results reveal strong peaks at about 0.5, 1.1 and 1.8 keV corresponding to signals from oxygen, sodium and silicon atoms, respectively. While Si and Na peaks originate from the glass substrate solely, O peak is coming from both substrate and oxide film. Analyzing relative intensities of Si and O peaks and comparing them between each other, it can be seen that the ratio between these peaks is different for TiO_2 and Al_2O_3 . This observation may indicate different distribution of the oxygen atom on the surfaces of oxides. Hence, it can be one of the reasons for the difference in the behavior of cells while they are seeded on the surfaces of TiO_2 and Al_2O_3 .

With EDS analysis, to estimate from which depth signal is actually originating, one need to take into account order of density values for material and accelerating voltage used for electron beam. Although, interaction volume of electron beam surely exceeds thickness of deposited thin films in our case, as it was mentioned above it still can give an idea about distribution and ratio of atoms. In order to obtain more reliable information about atoms distribution in thin films, substrate not containing atoms of O, Ti and Al could be used. It would exclude superimposing of the signals coming from substrate and oxide thin films. For this reason, such approach is preferential and used in many studies [11].

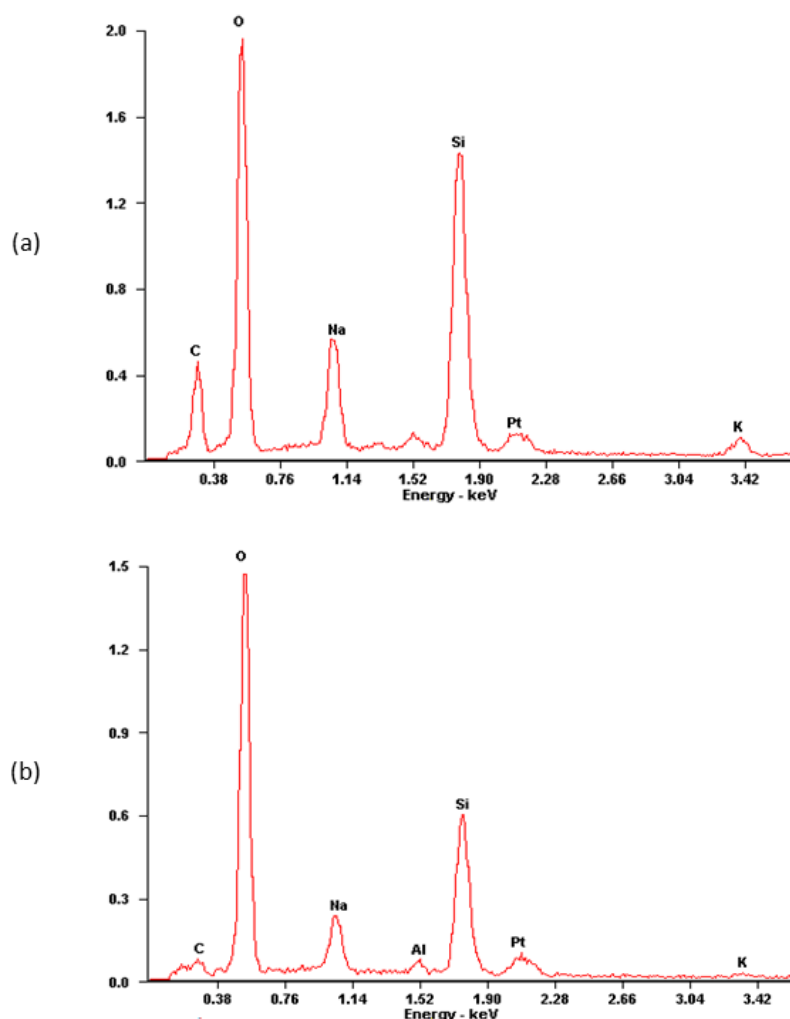


Figure 3.5. EDS pattern of TiO_2 (a) and Al_2O_3 (b) thin films deposited at 300 and 200°C, respectively.

3.1.6 Conclusion

As it is mentioned in the introduction of this subchapter, bio-response of the material is in strong dependence with its physicochemical properties. Therefore, it is important to start study with characterization of materials with cell-free surfaces, i.e. prior to any experiments involving cell seeding. A number of techniques have been used in order to get data about physical proprieties of deposited oxide coatings. In regards to chemical properties, EDS analysis did not provide explicit data on surface chemistry of oxides. Nevertheless, it allowed

to confirm successful deposition of thin films. Potentially, it can be applied also to get information about cell coverage area since this 'organic' part would give rise to signals from such atoms as carbon primarily. In order to have more detailed data on surface elemental distribution on bare thin films, methods like X-ray photoelectron spectroscopy (XPS) could have been good addition to characterization [12, 13].

Some material properties were not assessed in this work, though they can affect a cellular behavior. One of them being electronic properties of material surface. For example, some publications report the effect of electrical charge distribution on adhesion and proliferation of osteoblasts [14, 15]. In [14], osteoblasts' adhesion and proliferation was favored by negative charges generated on substrate surfaces by polarization. Interestingly, contrary to this observation, Dhayal et al. in [15] consider availability of exposed positive charges as key factor for improving adhesion and proliferation of osteoblasts. With this comparison, it is evident that the relation between material property and cellular response is not always clear and straightforward and often depends on a combination of factors. Response of MSCs depending on surface chemistry and deposition parameters of oxide thin films will be discussed in the following subchapters.

3.2 hBMMSCs adhesion and proliferation on thin films of TiO_2 , Al_2O_3 and VO_x

In this part of Chapter 3, results on influence of various parameters of deposition on the cellular behavior will be presented. At first, effect of oxygen pressure and temperature of deposition will be discussed. The point addressed afterwards is related to influence of thin films composition on cellular response.

3.2.1 *The influence of the parameters of deposition*

3.2.1.1 *Deposition in the O_2 atmosphere*

The influence of oxygen pressure was assessed by comparison between VO_x thin films deposited with and without O_2 gas atmosphere. Gas flow was inserted directly in the vacuum chamber during pulsed laser deposition. It is worth to note that introducing O_2 atmosphere leads not only to the increase of partial pressure by several orders of magnitude, but also to a possible change in stoichiometry of deposited oxides. Films of VO_x were chosen for these experiments for the latter reason. As it was mentioned in the first part of this chapter, vanadium can form oxides of various stoichiometry (Fig. 3.1a). Among principal oxides of vanadium are VO , V_2O_3 , VO_2 and V_2O_5 with oxidation states of vanadium equal to +2, +3, +4 and +5, respectively. Therefore, formation of vanadium oxides can be sensitive to the presence of O_2 atmosphere during synthesis of thin films [16].

Results obtained within our study indicate that presence of O_2 during deposition of VO_x on glass substrates did not lead to any effect that could be considered as significant (Fig. 3.6 and 3.7). Note that thin films of VO_x deposited at 500°C were not used in the study apart from the experiments about influence of the O_2 presence during deposition described above. This choice is due to the proximity of this temperature value to the melting temperature of aluminum (660.3°C), which is the material used for mask in sample holder in PLD system.

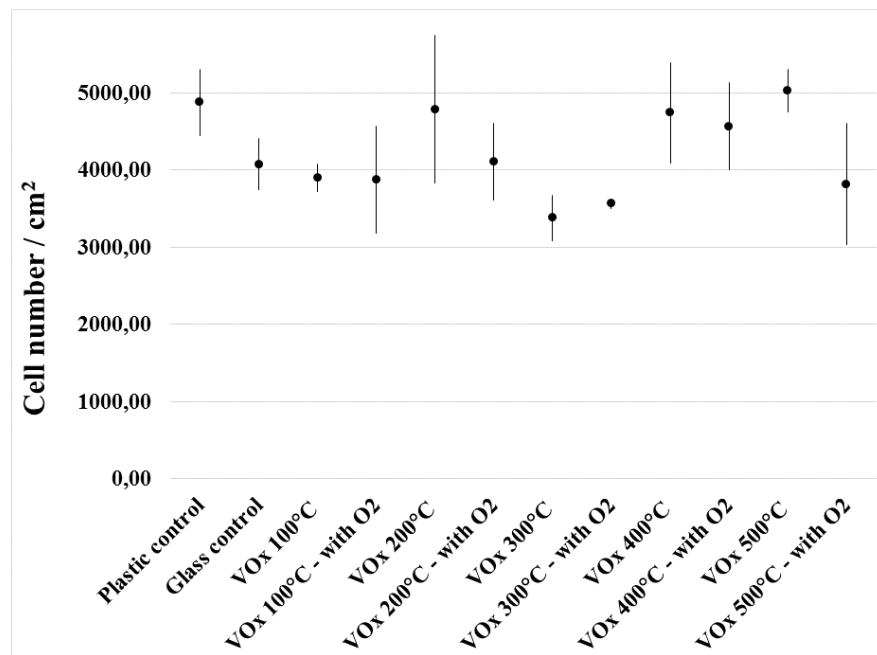


Figure 3.6. Cell adhesion after 2 hours of cell culture for controls and VO_x thin films deposited at various temperatures with and without O₂ gas flow.

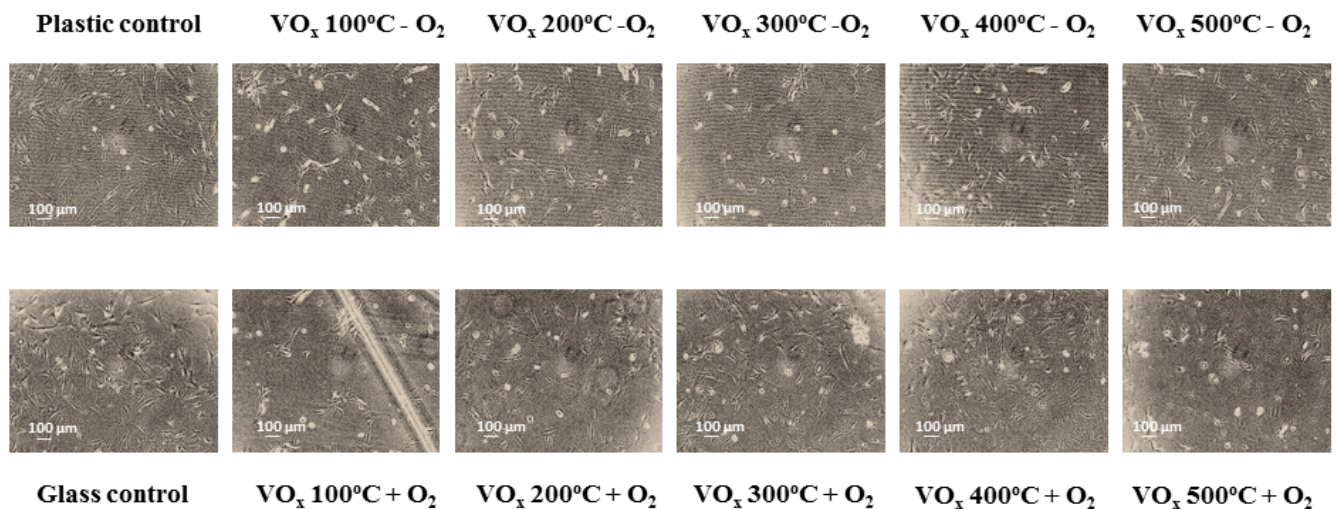


Figure 3.7. Phase contrast images of MSCs seeded onto VO_x thin films deposited at various temperatures with and without O₂ atmosphere after 24 hours of incubation.

Colorimetric WST-1 cell viability assay was used to know whether deposition under oxygen pressure leads to difference in the cytotoxicity of VO_x thin films. The results of WST-1 test (Fig. 3.8) after 48 and 72 hours of cell culture revealed that presence of O_2 gas flow during deposition of VO_x does not bring positive effect on the cell viability in comparison to deposition in normal atmosphere. In addition, it can be seen that VO_x probably have some cytotoxic effect on MSCs as comparing to glass control. This effect will be observed in results described further in this chapter, namely in the part concerning study of cell culture on oxide films deposited at same temperature (3.2.2.2).

Based on the results of WST-1 viability tests and phase contrast microscopy observations described above, deposition with O_2 gas flow did not bring visible positive effect on adhesion and proliferation of mesenchymal stem cells. Therefore, it was decided to continue thin films' synthesis in normal conditions, i.e. without introducing oxygen gas flow in the vacuum chamber during deposition.

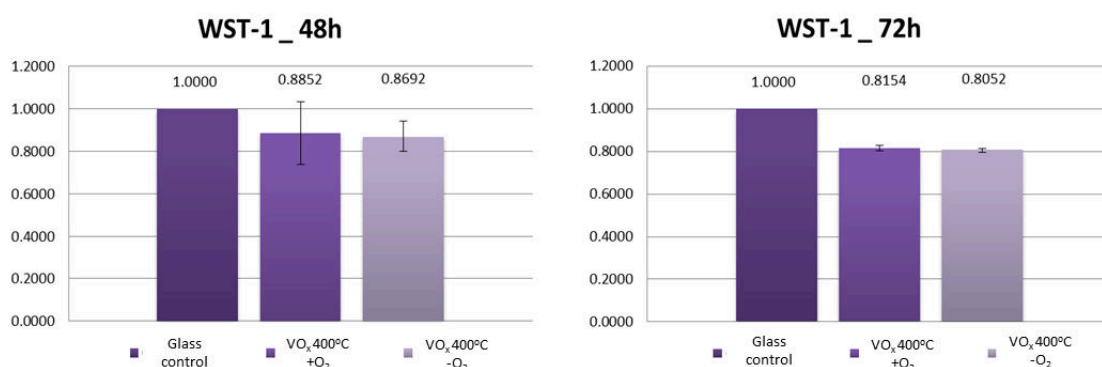


Figure 3.8. Results of WST-1 test for MSCs seeded onto VO_x thin films deposited at 400°C with and without O_2 atmosphere.

3.2.1.2 Effect of different deposition temperature

In order to study whether the deposition temperature affect hBMSCs adhesion and proliferation, cells were seeded onto thin films of TiO_2 , Al_2O_3 and VO_x deposited at 100, 200 and

300°C and control glass substrates. In addition, TiO_2 and Al_2O_3 films of different thickness were used in order to test whether this parameter has an effect on cell response.

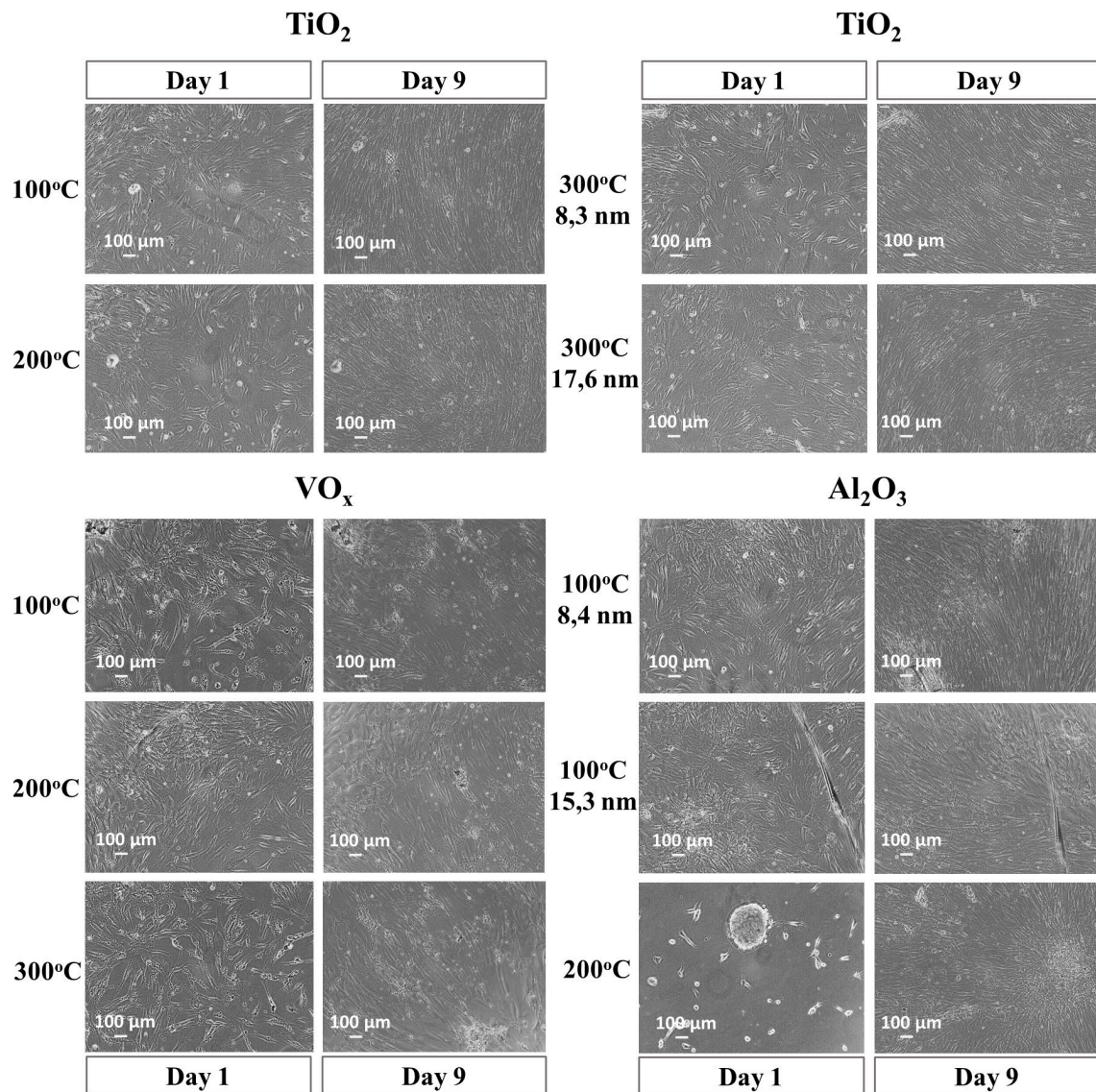


Figure 3.9. Phase contrast images of MSCs seeded onto: TiO_2 (top) thin films deposited at 100, 200°C, and at 300°C with different duration of deposition; VO_x thin films deposited at 100, 200 and 300°C (bottom left); Al_2O_3 films deposited at 100°C with different duration of deposition and at 200°C (bottom right).

According to the analysis of phase contrast microscopy images (Fig. 3.9), no remarkable difference in cell adhesion and proliferation was observed between TiO_2 oxide thin films

deposited at different temperatures. Similar to TiO_2 , no drastic change in cell behavior was noted between cells seeded on Al_2O_3 and VO_x films deposited at 100, 200 and 300°C (Fig. 3.9, bottom). Note that these temperatures are significantly lower than those utilized in some similar studies, there was also no annealing step and no additional heat treatment was carried out after deposition [17].

While temperature of deposition and presence of oxygen flow during deposition may play a big role in changes of crystallinity and surface chemistry of thin films, it seems that it did not affect dramatically cell adhesion and proliferation. Therefore, the discussion can be focused solely on the effect of surface chemistry of oxide thin films deposited within this study. The first part of the tests was concentrated on using substrates coated with thin films of Al_2O_3 , TiO_2 and VO_x deposited at 200, 300 and 400°C, respectively. Oxide thin films deposited at these particular deposition temperatures were chosen as they showed more promising results during preliminary experiments.

3.2.2 The influence of the thin film composition on cellular adhesion and proliferation

3.2.2.1 Cell culture on oxide thin films deposited at different temperatures

As mentioned above, initially for the *in vitro* experiments we used thin films deposited at particular temperatures. The choice was based on the preliminary tests with mesenchymal stem cells.

Human bone marrow mesenchymal stem cells (hBMMSCs) were seeded onto TiO_2 and Al_2O_3 thin films, as well as glass cover slips (no coating deposited), a reference, and monitored after 2 hours (for adhesion) and after 5 days of cell culture. Two hours after seeding hBMMSC displayed a rounded shape (Fig. 3.10a, top). According to our phase contrast microscopy observations, out of all samples, more cells were attached to the glass (1600 cells/cm²) at the very beginning of the culture. Comparing to this control sample, the adhesion efficiency (Fig. 3.10a, bottom) is 1.33 times lower for hBMMSCs seeded on TiO_2

film (1200 cells/cm²) and approximately 2 times lower on Al₂O₃ film (800 cells/cm²). This difference adhesion between both oxides remains present in the early stages of cell culture. It is attributed to the lower contact angle values for glass substrate and titanium oxide, which obviously facilitates the cells adhesion at least in the initial step of the growth [18].

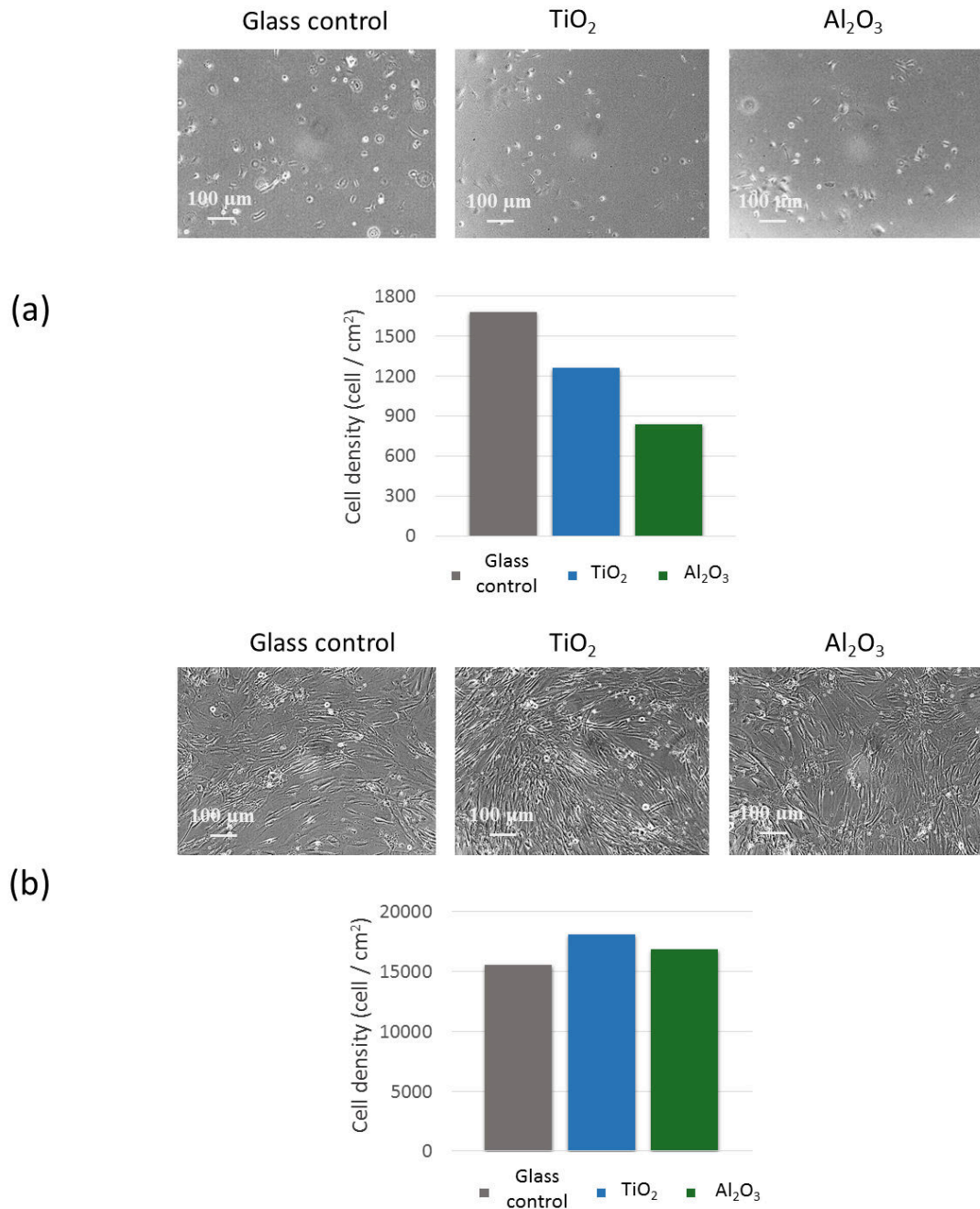


Figure 3.10. Optic microscopy images and corresponding cell density after 2 hours (a) and 5 days (b) of cell culture

After 5 days of culture (Fig. 3.10b, bottom), the situation is however different. The cell density is indeed approximately 14% higher on TiO_2 film (18,000 cells/cm²) and 7.5% on Al_2O_3 film (16,000 cells/cm²), compared to the glass control (15,500 cells/cm²). Also, the mesenchymal stem cells cultured on both thin films, as well as on glass control (Fig. 3.10b, top), displayed an elongated shape, with no preferential orientation. These results reveal that, during the first stages, the cell growth somehow depends on the surface properties such as composition and wettability, however the effects also depend on the time of the culture.

The evolution of cell density was further analyzed for the whole duration of the experiment, and the results are displayed in Fig. 3.11a. After 24 hours, the cell density on TiO_2 exceeds the one on glass control, while it took 48 hours for Al_2O_3 to surpass control sample in cell density. This difference with respect to the reference sample keeps increasing as a function of time. Therefore, the population growth velocity becomes higher on the oxide films rather than on glass control, confirming the importance of the oxide coating composition. This can be seen by the evaluation of cell population doubling time (Fig. 3.11b), which is higher on glass control.

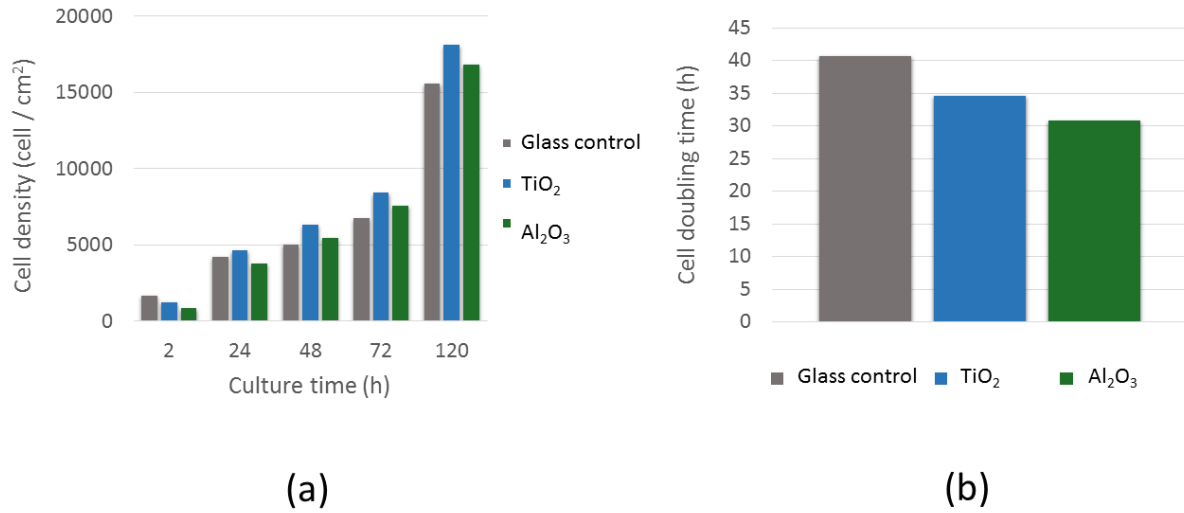


Figure 3.11. Cell density vs culture time for cell cultured on glass substrate and TiO_2 , Al_2O_3 thin films (a); corresponding cell doubling time for glass control and TiO_2 , Al_2O_3 thin films (b).

The final higher cell density on thin films samples can be explained by the fact that

starting from the first days of culture, the number of hBMMSCs adherent on titania surface was higher than that on the glass control, while it took two days for cells cultured on alumina surface to surpass the number of cells measured on control glass. Moreover, the value of cell population doubling time is lower for alumina surface (27.3 hours) than on titania surface (30.7 hours), and glass control (36.8 hours). Considering the initial density of adherent cells on each condition, this indicates that the growth of hBMMSCs is improved significantly in contact with Al_2O_3 film, rather than with TiO_2 film or uncoated glass (Fig. 3.11b). Overall, this suggests that both TiO_2 and Al_2O_3 compositions may have a catalytic action (with a slightly more favorable effect on Al_2O_3) on the development of these stem cells, and that the hydrophilicity of the surface is essentially important in the stem cell development, especially at the very beginning of cell culture. Similar to titania and alumina, vanadium oxide thin films deposited at 400°C exhibited good performance in terms of cell adhesion and proliferation, as cell density and morphology matching the ones on the glass control samples can be observed (Fig. 3.12).

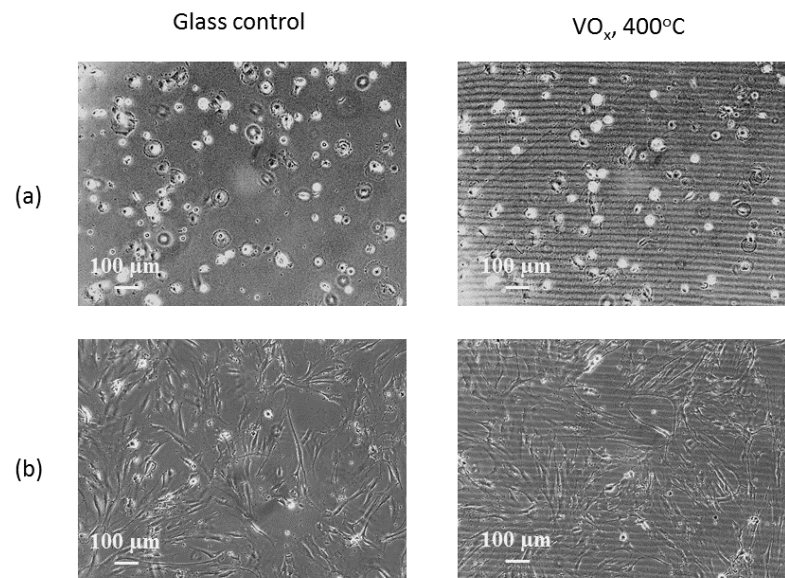


Figure 3.12. Optic microscopy images of cells on glass control and VO_x thin films after 2 hours (a) and 3 days (b) of cell culture.

The organization of the cell was also studied (Fig. 3.13). After 16 days of culture on glass control, the stem cells adopt a circular alignment to form a multicellular structure (Fig. 3.13d). This cell conformation is already displayed by hBMMSCs cultured on both oxide thin films after only 6 days (Fig. 3.13b and c), contrarily to the glass control (Fig. 3.13a).

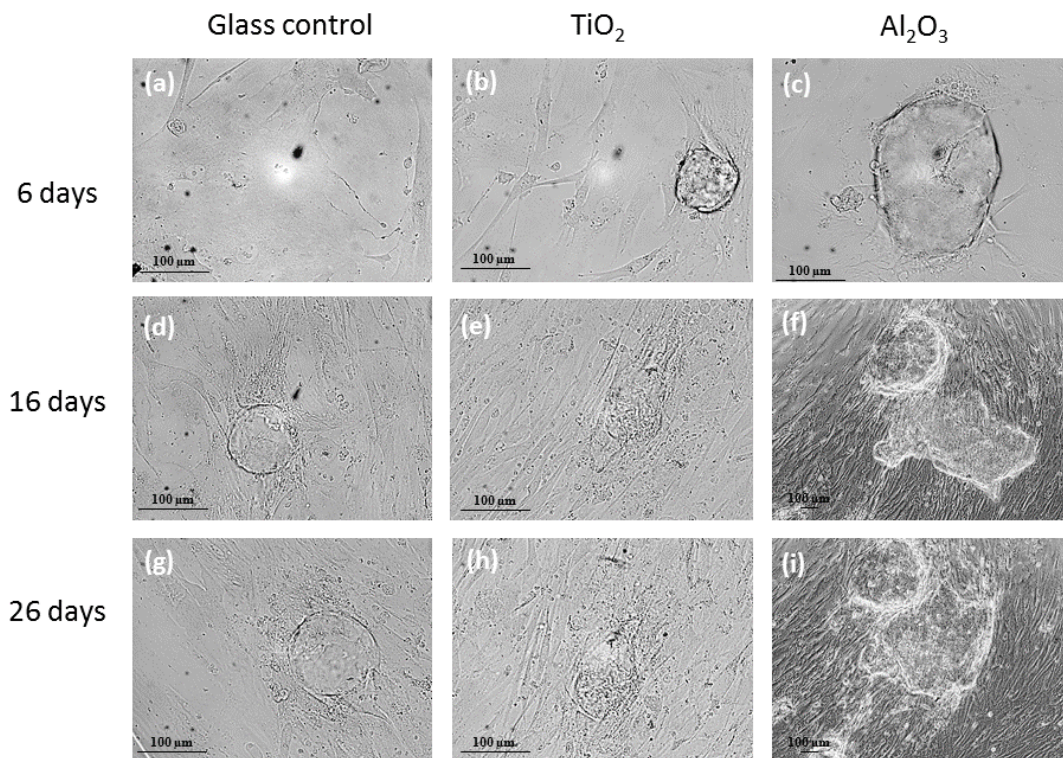


Figure 3.13. Phase contrast optical microscopy images of BMMSCs on glass control (a, d, g) , TiO_2 and Al_2O_3 thin films (b, e, h and c, f, i, respectively) after 6, 16 and 26 days of cell culture.

This type of multicellular condensation was already reported as the natural starting-point of chondrogenesis and osteogenesis differentiation [19]. Several research groups have induced such cell aggregation, using other source of cells, as adipose-derived stem cells [20, 21] and various approaches, as electrical field [22] and nanoscale features [23]. In our work, alumina surface seems the most effective in terms of formation of this multicellular condensation, considering its initial and final size after 26 days of culture (Fig. 3.13i), in comparison to

the glass control and titania surface (Fig. 3.13g and h, respectively). This phenomena was also observed on VO_x thin films during *in vitro* tests concerning effect of oxygen gas presence in the PLD process (Fig. 3.14).

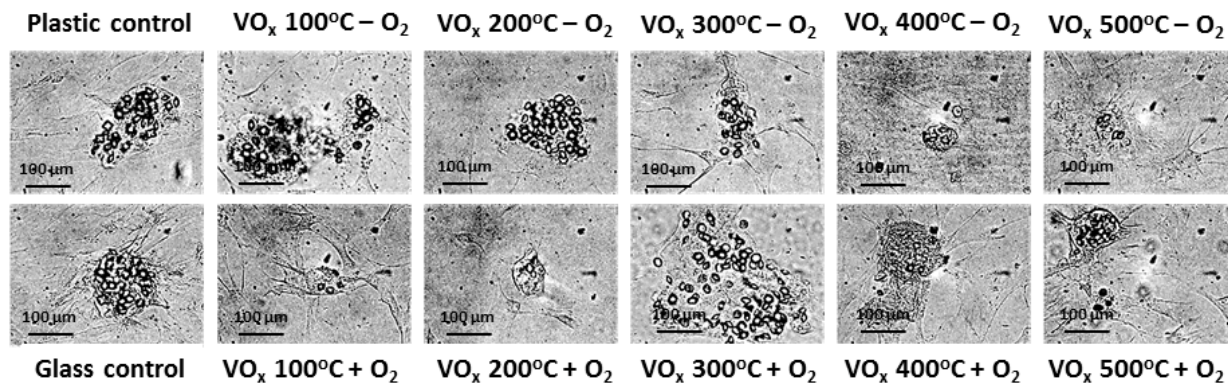


Figure 3.14. Phase contrast images of MSCs seeded onto VO_x thin films deposited at various temperatures with and without O_2 atmosphere after 2 days of incubation. (40x magnification)

These multicellular structures were analyzed by means of scanning electron microscope (Fig. 3.15). Matrix deposition by cells was observed in all conditions, but the more significant improvement in fibers density seems to be observed on alumina surface (Fig. 3.15b). It is worth to note that matrix deposition is higher on glass control than on TiO_2 film, which means it follows the same trend previously observed for rate of structure formation (Fig. 3.13). Fibronectin deposition during hBMMSCs aggregation has already been reported, suggesting that this extracellular matrix protein plays a role in the cell condensation phenomenon [24]. In our case, further studies would be valuable to know whether different oxide thin films lead to the matrix deposition of different composition. Complementary information could be obtained by the evaluation of the ability for oxide thin films to bind specific proteins provided by the culture media. For example, compared to graphene, the higher adsorption capacity of graphene oxide for serum proteins resulted in a higher density of adhesion sites available for cell attachment and growth [25]. Furthermore, it was reported

that osteogenic differentiation of hBMSCs was enhanced by the capacity of graphene to fix β -glycerophosphate and dexamethasone, while the adipogenic specialization was improved by the ability of graphene oxide to fix insulin. Compared to the glass control, the acceleration of hBMSCs growth and condensation could be explained by a potential difference related to the proteins adsorption ability of particular oxide thin film.

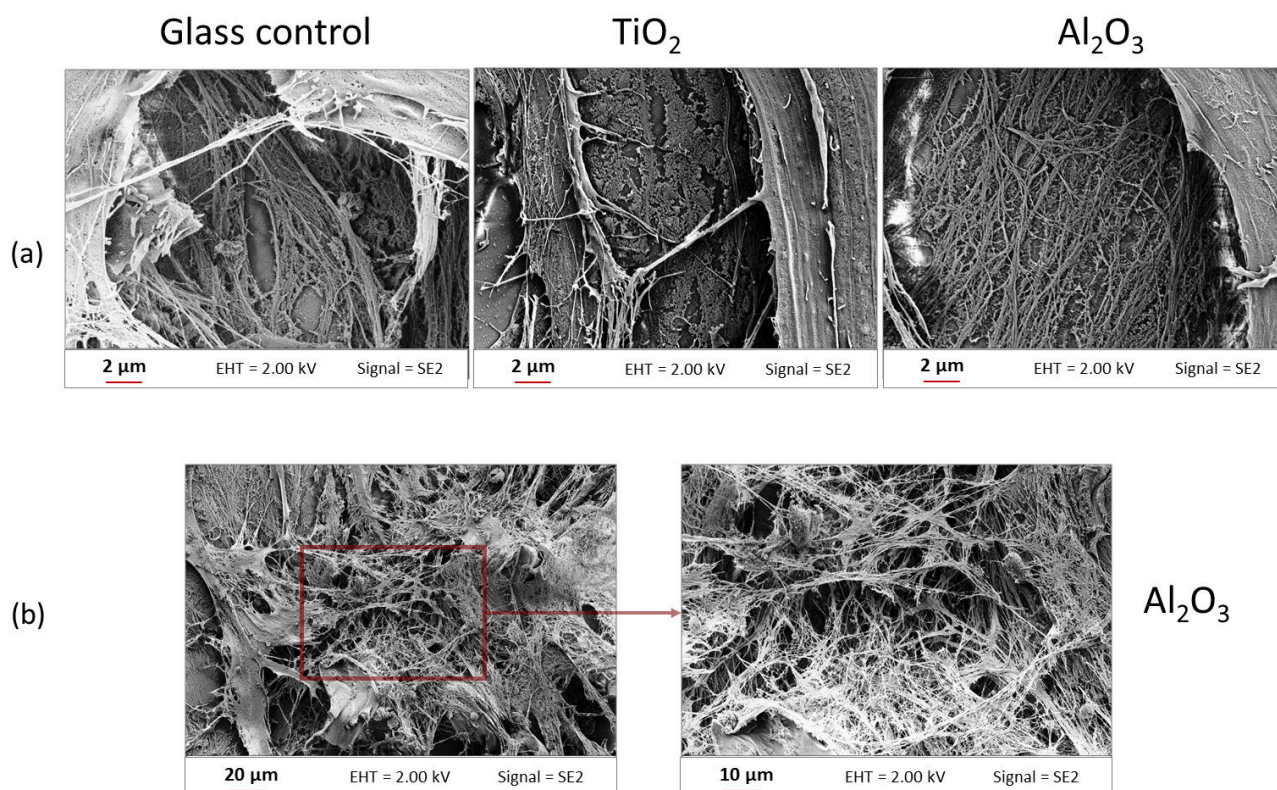


Figure 3.15. SEM images of BMSCs on glass control, TiO_2 and Al_2O_3 thin films after 14 days of culture (a), SEM images of multicellular structures on Al_2O_3 (b).

To the best of our knowledge, the potential impact of oxides thin films on this kind of hBMSC condensation was not explored before. Appearance of multicellular structures could be investigated more thoroughly as it is possibly influenced by the surface chemistry. However, this observation was not exploited further since it was considered to be out of scope of the current study.

Set of experiments conducted during first part of my study was aimed at determining composition of thin films, as well as optimal conditions for deposition and cell culture. It is worth to note, although the results described above represents quite well the tendency of cell adhesion and proliferation on TiO_2 and Al_2O_3 thin films, some data lacks statistics and was meant to help planning following experiments. In regards to vanadia films, results obtained within second part of my study were somehow different from discussed above, possible explanation consists in using of two different sources of MSCs for *in vitro* tests (mesenchymal stem cells from two donors), and this observation will be discussed in the next subchapter.

Nevertheless, it can be seen that these preliminary set of data lead to the evident conclusion of possibility of using metal oxide thin films deposited by PLD technique as substrate coatings for studies related to hBMMSCs.

3.2.2.2 Cell culture on oxide thin films deposited at the same temperature

In this part, results on *in vitro* stem cell cultures with TiO_2 , Al_2O_3 and VO_x thin films deposited at the same temperature, namely 300°C , will be discussed. Such comparison allows to minimize all differences between oxides related to deposition parameters. At first, results on TiO_2 and Al_2O_3 will be described. Afterwards, effect of VO_x thin films deposited at 300°C will be presented.

To study influence of TiO_2 and Al_2O_3 on cell adhesion and proliferation, human bone marrow mesenchymal stem cells (hBMMSCs) were grown onto oxide thin films, as well as glass cover slip (no coating deposited) served as reference. Two hours after seeding on glass control and both thin films, rounded shape of hBMMSCs can be observed (Fig. 3.16a). Calculations of adherent cells number indicate that no significant difference between substrates coated with oxide thin films and glass control can be noted (Fig. 3.16b).

As described in the first chapter, cell adhesion is complicated process that is sensitive to a number of factors [26]. Assuming that protein adsorption plays a key role in cell adhesion, special attention should be paid to the surface properties that have an impact

on the protein-surface interaction. One of them being surface wettability that was assessed during present work. Hydrophobic surfaces is believed to favor adsorption of proteins in general. However, in the case of glycoproteins, among which there are such as fibronectin, cadherin and integrin, there is an affinity towards hydrophilic nature of surface. Namely mentioned proteins play a major role in mediating adhesion process [27, 28].

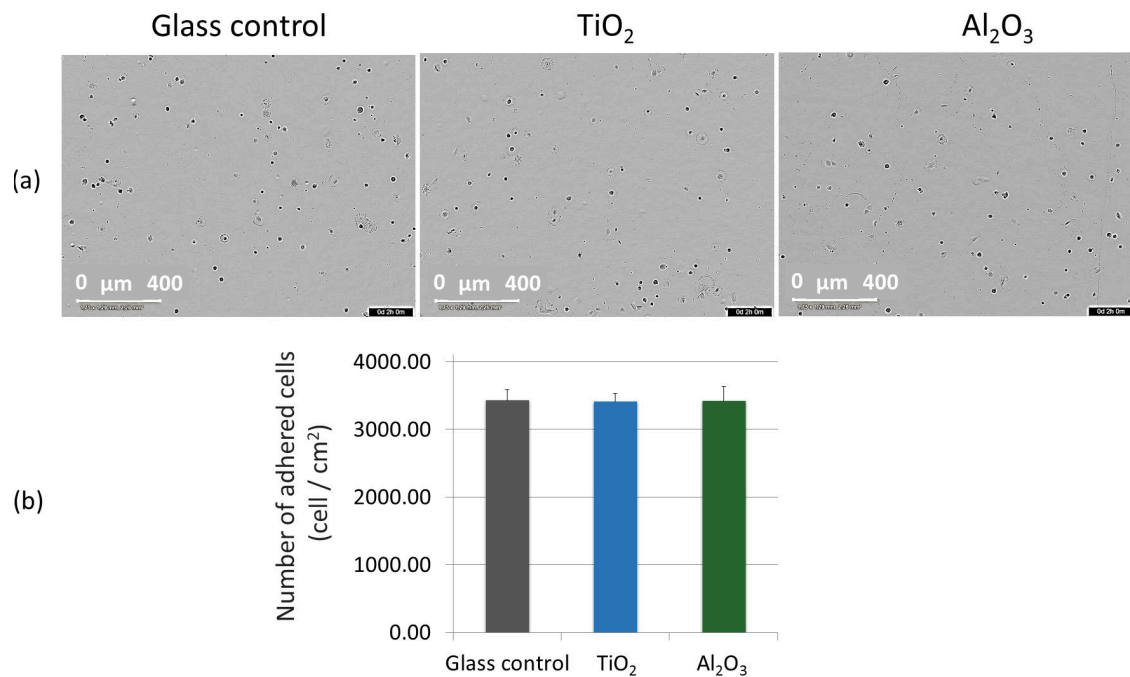


Figure 3.16. Optic microscopy images of BMMSCs on glass control, TiO₂ and Al₂O₃ thin films after two hours of cell culture (a); corresponding cell density (b). No significant difference between glass control and coated substrates is observed indicating normal adhesion for oxide thin films.

Taking the above into consideration, in regards to cell proliferation, higher number of cells on glass control and TiO₂ (Fig. 3.17) is considered to be related to the reported lower contact angle values facilitating cell adhesion [18]. It can be also seen that both oxides did not interfere with normal division in the early stages of cell culture as number of cells after 2, 4 and 7 days was similar for all studied samples. Despite using two different cell sources for these measurements, similar trend as in the case of discussing films deposited at different temperature (part 3.2.2.1) can be observed.

It is worth to note that in order to obtain data for proliferation curves, cells were harvested by trypsinization. This way, cells are collected from entire surface, which includes cells grown on the part of plate well not covered with glass slide, i.e. plastic. One of the methods to overcome this issue is fluorescence that is widely used in biological research. Using appropriate fluorescence dyes allows to stain cellular components and monitor not only cell division and proliferation, but also such dynamic changes in cells as intracellular transport and secretion [29, 30].

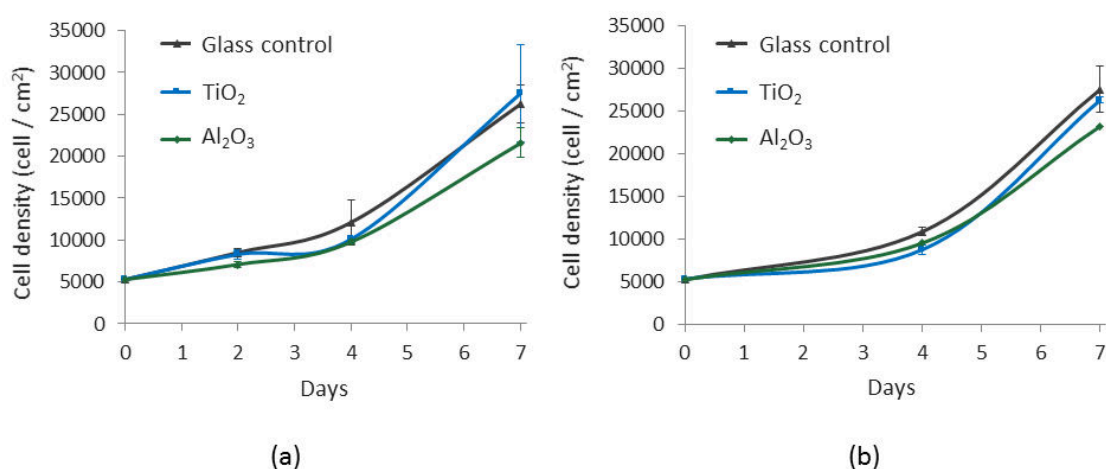


Figure 3.17. Cell density BMMSCs cultured on glass control, TiO₂ and Al₂O₃ thin films in the initial point of cell culture and after 2, 4 and 7 days (a); initial point and after 4 and 7 days of cell culture (b). Note that curves on (a) and (b) were calculated for MSCs obtained from two different sources.

After initial stage of adhesion, MSCs start to proliferate and within first two days of culture they start to display an elongated shape with no preferential orientation. Within this study, staining of Van Gieson, hematoxylin phloxine saffron and Masson's trichrome were used to highlight cell morphology (Fig. 3.18). Mesenchymal stem cells that were cultured for 14 days on both thin films, as well as on glass control, exhibited spindle-like shape which is typical for this cell type. In addition, uniform cell organization was noted with no

significant inhomogeneity in cell spreading between different samples. It was demonstrated in some studies [31], including that of McMurray et al. [32] that morphology can direct a differentiation path of a stem cell. Therefore, maintaining of the morphology associated with undifferentiated state of MSCs can be very important in terms of their differentiation potential [33].

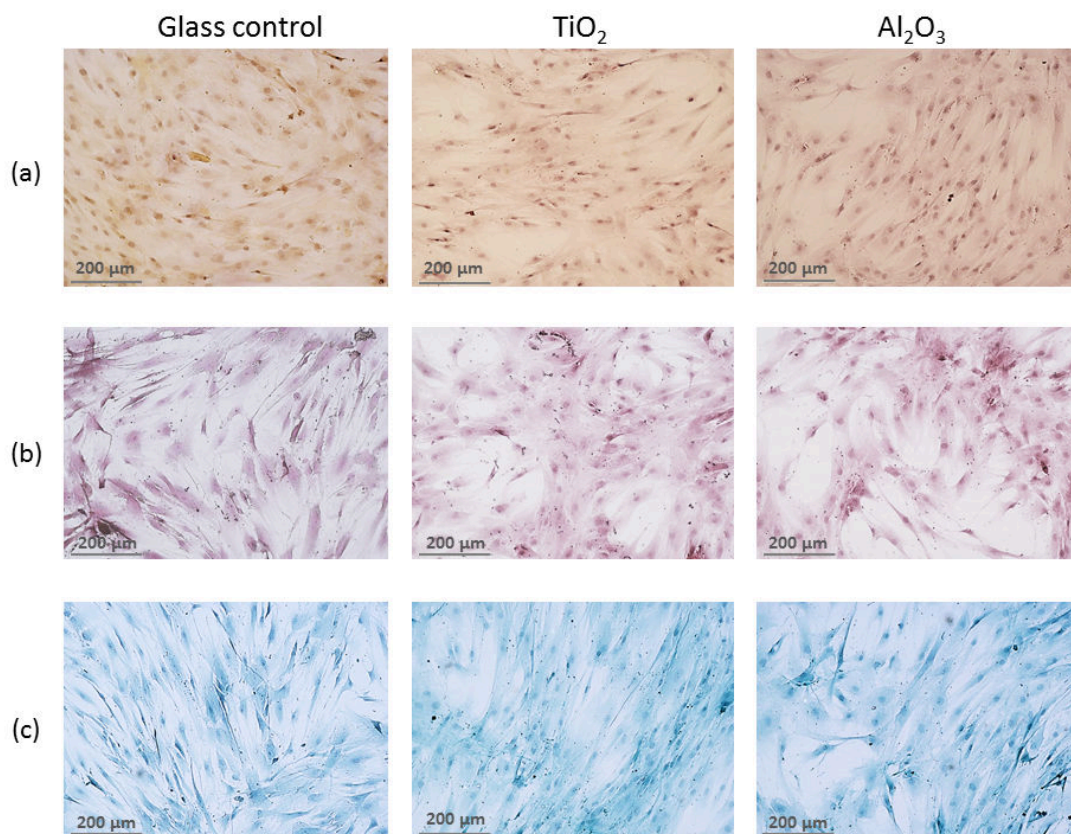


Figure 3.18. Phase contrast optical microscopy images of Van Gieson (a), hematoxylin phloxine saffron (b) and Masson's trichrome(c) staining after 14 days of cell culture on glass control, TiO₂ and Al₂O₃-coated substrates. All three staining methods indicate healthy morphology of cultured MSCs.

The evolution of cell density and growth was further analyzed by means of scanning electron microscopy (Fig. 3.19). After 7 days of culture, flattened MSCs of size in the range of 10 - 20 μm can be distinguished on the glass control and TiO₂, Al₂O₃ thin films (Fig. 3.19a, b, c). For cells after 14 days of culture (Fig. 3.19d, e, f), SEM images were acquired

with another type of detector (for backscattered electrons), which allowed to obtain more clear view on extracellular matrix. Deposition of matrix was observed for all conditions with similar cell organization as it can be seen that cells on both oxides and control glass adopt a linear alignment.

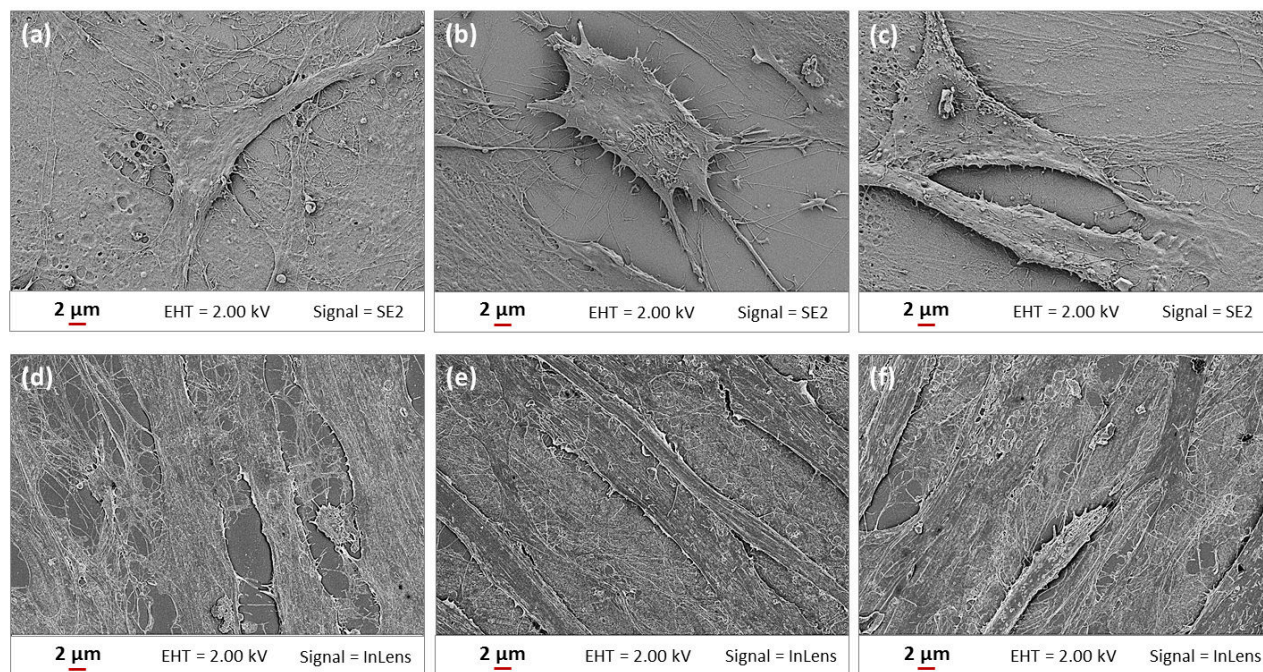


Figure 3.19. SEM images of glass control (a,d), TiO₂ (b,e) and Al₂O₃-coated (c, f) substrates after 7 days (top) and 14 days of cell culture (bottom).

In regards to MSCs culture on VO_x thin films, as it can be seen in Fig. 3.20a, fewer cells were attached to the surface of the thin film in comparison to the glass control. After 10 days of cell culture, this trend of different cell amount continues and cells cultured on the control had higher density that can be observed in Fig. 3.20b. Judging by images obtained with scanning electron microscopy (Fig. 3.21), VO_x thin films also have an impact on morphology of cells. Comparing to cells on glass control, MSCs cultured on oxide thin film appear more narrow, with long filopodia, what can be considered as mild deviations from healthy morphology [34].

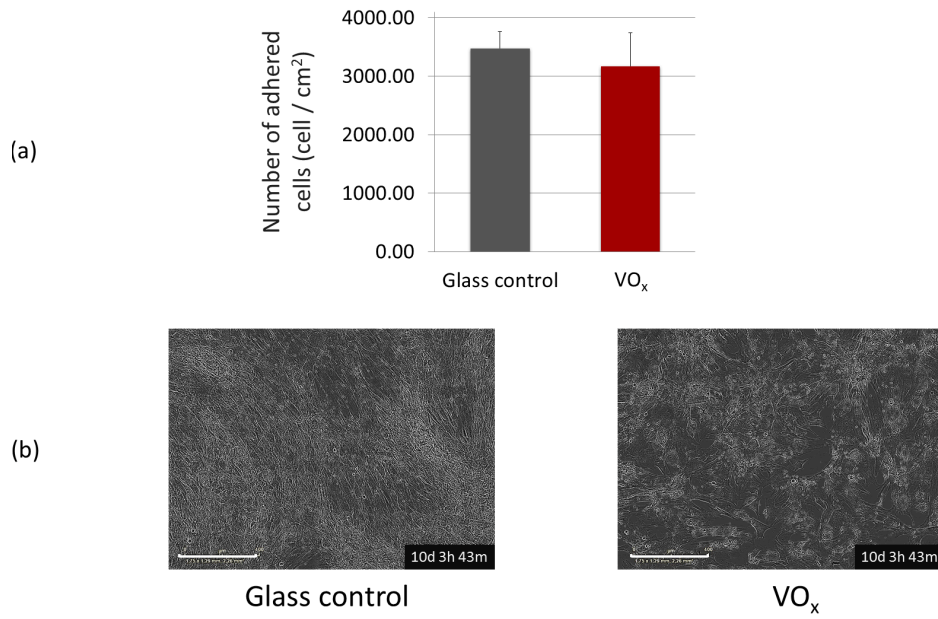


Figure 3.20. Cell density of hBMMSCs cultured on glass control and VO_x thin films after 2 hours of culture (a) Phase contrast images of hBMMSCs on same substrates after 10 days of culture with visible difference in amount of cells (b). Scale bar = 400 μm .

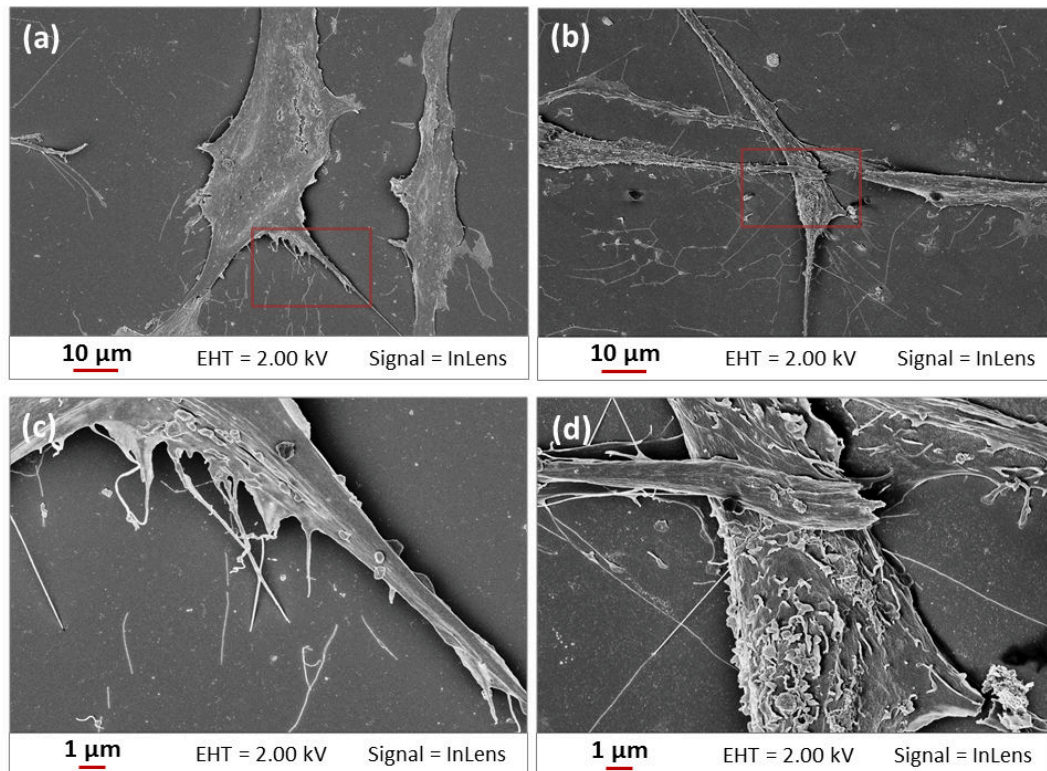


Figure 3.21. SEM images of glass control (a,c) and VO_x-coated (b, d) substrates after 7 days of cell culture with magnification of $\times 1000$ (top) and $\times 5000$ (bottom).

These results indicate inhibitory effect of VO_x thin films on the proliferation of hBMMSCs. Such quality can be interesting for investigations related to stem cell behavior and especially to the cancer stem cells, namely for searching ways to stop the development and growth of tumors [35]. Interestingly, in contrast to mesenchymal stem cells, VO_x thin films had the opposite effect on cell growth in case of some types of cancer cells (will be discussed further in subchapter 3.4).

To the best of our knowledge, there are no reports on oxides of vanadium used for human mesenchymal stem cell culture. However, Srivastava et al. studied the effect of vanadium (IV) oxide on mediation of osteoblast differentiation of mouse mesenchymal stem cells [36]. Oxide of vanadium, namely in the form of nanotubes, has been also identified as cytotoxic to human colon carcinoma cells. Rhoads et al. showed these cells undergo loss in viability after few hours of being exposed to vanadium oxide nanotubes [37].

3.2.3 Conclusion

In this subchapter it was shown how oxide thin films can be used for culture and study of human mesenchymal stem cells. TiO_2 , Al_2O_3 and VO_x thin films were successfully deposited by pulsed laser deposition technique and their impact on adhesion and proliferation of hBMMSCs was evaluated. From our results, it can be concluded that TiO_2 and Al_2O_3 thin films allow MSCs to adhere, grow and proliferate in good states. For TiO_2 , Al_2O_3 , any slight difference in outcome of experiments can be explained by the difference in their wettability, which is considered as the surface property with significant influence on cell behavior. Hydrophilic surfaces are believed to be preferential for cells in general [38]. However, despite the fact that surface of VO_x is more hydrophilic than surface of TiO_2 and Al_2O_3 , apparently, it causes inhibition of MSCs proliferation. Therefore, this observation can be explained only by the effect of surface chemistry. Taking into account the fact that we used thin films with similar roughness of 0.1-0.3 nm, it can be expected that wettability of films also depends mostly on its chemical composition. In regards to vanadium oxide, one of

the possible explanations of its cytostatic effect on MSCs is the release of soluble vanadium derivative upon contact with culture medium. For example, high solubility of vanadium pentoxide (V_2O_5) can cause a toxic effect due to the release of vanadate ions [39]. However, some studies have demonstrated that at relatively low concentrations of these released ions (up to $10\ \mu\text{M}$), vanadium compounds can be efficient for cell growth improving [40, 41].

In addition to the main set of *in vitro* experiments, thin films of CuO, MgO and ZnO deposited at 100°C were used to estimate their interaction with MSCs. As it can be seen in Fig. 3.22a, there is a considerable difference in the amount of cells after 4 days of culture between CuO and two other thin films. Furthermore, peculiar cell morphology with spherical features was observed on CuO, which could be also interesting to explore. These results further showcase the benefits of having a wide variety of compositions of oxide films, which allows to obtain complementary information regarding influence of surface chemistry on cellular response within the framework of a single study.

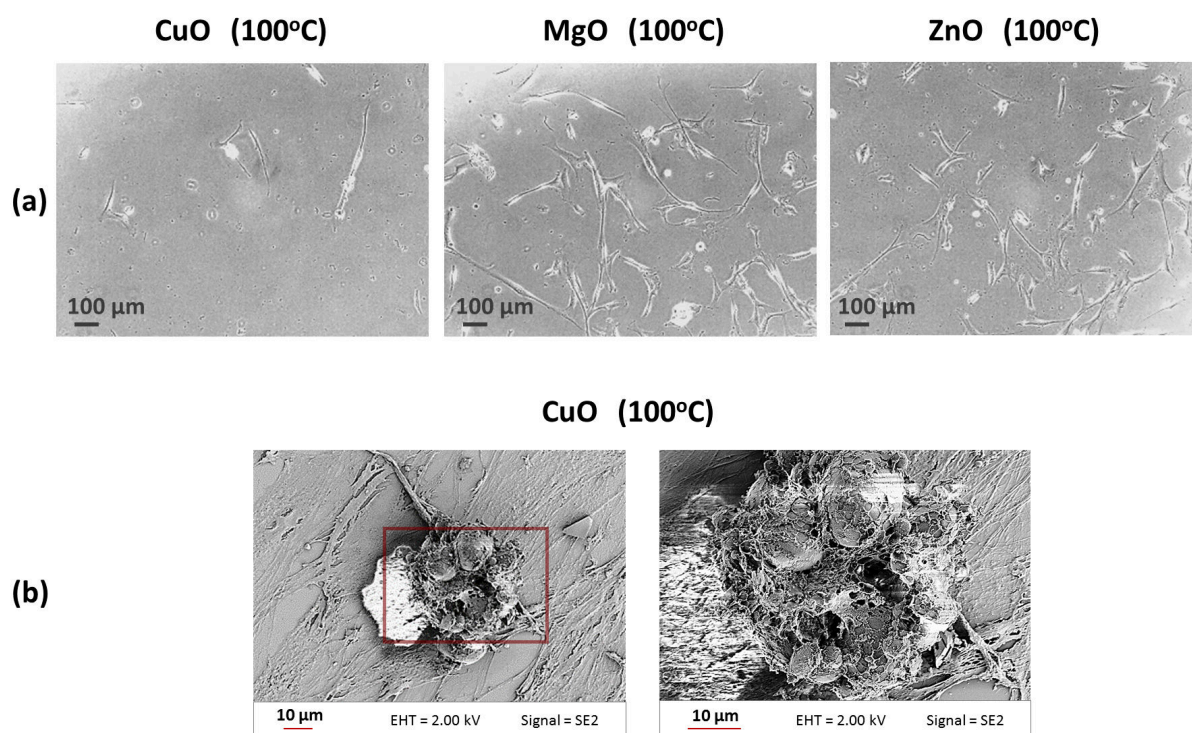


Figure 3.22. Phase contrast images of MSCs after 4 days of culture on CuO, MgO and ZnO thin films deposited at 100°C (a), SEM images of cells grown on CuO thin films (b).

In general, interaction between material and stem cells can be influenced by many parameters. Talking about material properties, there have been a number of reports proving that surface chemistry, roughness and wettability, they all play a major role in affecting cell response. Dubey et al. illustrated that surface charge is among other factors that have an effect on cell behavior [42]. They showed how applying electrical pulses can stimulate cell growth and proliferation. Some studies have identified plating density and time in culture as parameters that can affect cell proliferation and characteristics [43–45]. In addition, different type of cells can behave in a different way on the same surface. For example, being polar, fibroblast-like cells, in comparison to non-polar epithelial-like cells, possess higher dependency on surface chemistry with possible abrupt transition [46].

There have been studies demonstrating that relation between surface properties and cellular response is not always straightforward [47]. Overall, it is more likely that in order to get the best result in terms of biomaterial performance, suitable combination of mentioned above properties is usually the most efficient. For example, Vandrovcova et al. showed how adhesion, growth and phenotypic maturation of MG63 cells were controlled by the interplay between the material chemistry and surface topography [48]. To conclude, while a lot of the recent studies concentrate on using nanomaterials or materials with modified or treated surface, our goal was to demonstrate that using simple binary oxide thin films can help in understanding the effect of chemistry on the cellular response.

3.3 hBMMSCs differentiation on thin films of TiO_2 , Al_2O_3 and VO_x

In this part of the chapter, the results on differentiation of hBMMSCs seeded on the oxide thin films are described. Differentiation was monitored by the means of histological staining and real-time reverse transcription polymerase chain reaction (RT-PCR). Analysis of cell differentiation, even if it is not in the final stage, allows to obtain information about stem cell fate, i.e. cell predisposition to become a certain type of cell. In the case of human bone-marrow derived mesenchymal stem cells, their differentiation potential into osteocytes (bone cells) and chondrocytes (cartilage constituting cells) is particularly interesting in terms of regenerative medicine and tissue engineering. Therefore, we focused namely on the analysis of osteogenic and chondrogenic differentiation of MSCs. In addition, their adipogenic potential was also assessed.

3.3.1 Differentiation staining

Evaluation of TiO_2 , Al_2O_3 and VO_x thin films influence on MSCs differentiation was done by various staining assays. In order to estimate impact of oxide films on osteogenic potential of hBMMSC, cells were cultured in osteogenic medium and Alizarin red staining that allows to mark calcium-rich deposits was used. Although such calcium-rich sites of mineralization were not detected in large quantities by optical microscopy observations, staining of cells which were grown on Al_2O_3 appear to be more pronounced than for those on TiO_2 or glass control (Fig. 3.23). It is worth to note that cell culture with VO_x thin films was carried out separately, what resulted in the slight difference between two sets of staining experiments. Nonetheless, it can be clearly seen that comparing to glass control, VO_x is showing reduced level of Alizarin red staining (Fig. 3.23b).

In regards to the other features of osteogenesis, change to polygonal shape that is typical for cells undergoing differentiation to osteoblasts could be partially seen for cells cultured

on Al_2O_3 . Overall, lack of pronounced signs of osteogenesis can be explained by insufficient duration of culture with differentiation medium. In some studies it was reported that more evident transformation of MSCs into osteoblasts can be reached with cell cultures during 21 days [49].

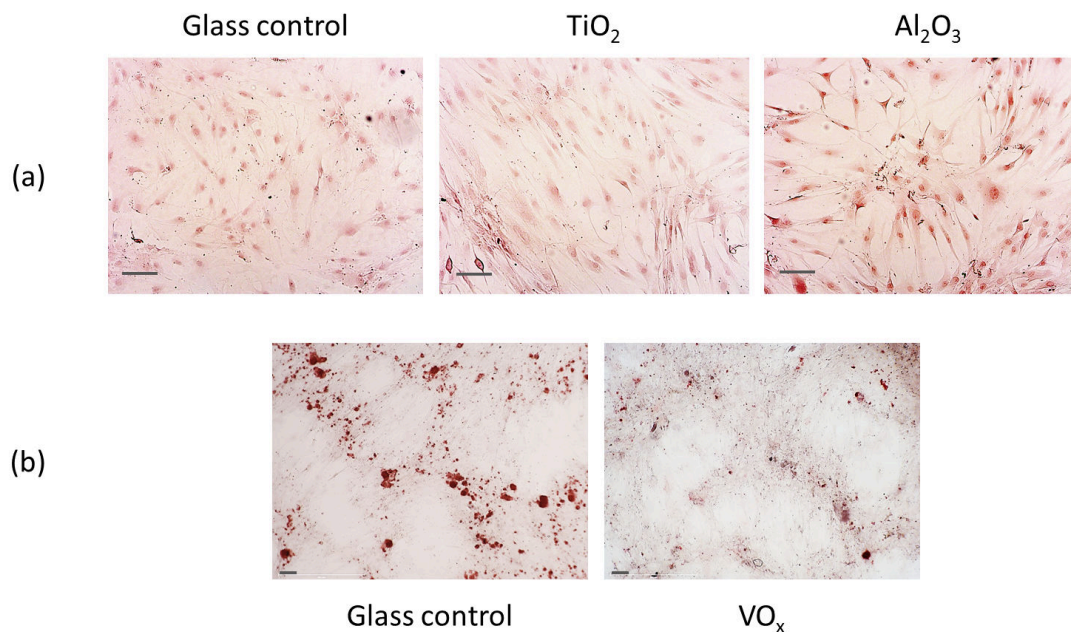


Figure 3.23. Alizarin red staining of (a) MSCs seeded on glass control, TiO_2 and Al_2O_3 -coated substrates after 14 days of cell culture and (b) MSCs seeded on glass control and VO_x in separate experiment. Scale bar = 100 μm .

With respect to influence of TiO_2 , Al_2O_3 and VO_x thin films on chondrogenic differentiation, MSCs after culture in chondrogenic medium were used for Alcian blue staining. Results of quantification of this staining by spectrophotometry are represented in Fig. 3.24. Taking into account standard error, effect of oxide thin films on the initiation of chondrogenesis can be observed. In particular, Al_2O_3 showed higher values for absorbance of eluted Alcian blue meaning positive influence of this oxide on chondrogenic differentiation of MSCs.

As far as we aware, effect of Al_2O_3 and VO_x on MSCs chondrogenic differentiation was not previously assessed in any reports. However, there are a few studies concerning effect of

TiO₂ on chondrogenic differentiation of MSCs [50, 51]. For example, Kaitainen et al. employed TiO₂ coatings to show how they can improve hMSCs proliferation without negatively affecting cell capacity fro chondrogenic differentiation [51].

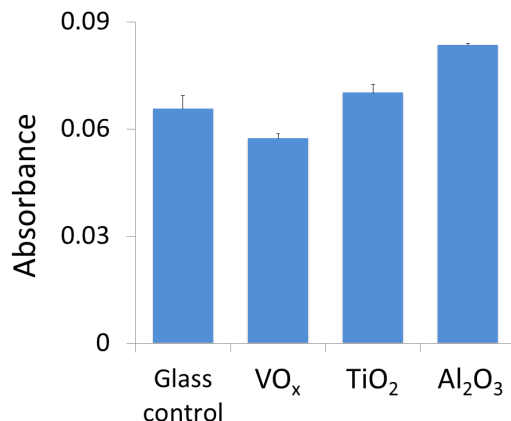


Figure 3.24. Absorbance of the eluted Alcian blue for MSCs seeded on glass control, VO_x, TiO₂ and Al₂O₃-coated substrates after 14 days of cell culture. Taking into account standard error, results indicate positive influence of Al₂O₃ and VO_x on chondrogenic differentiation of MSCs.

To visualize lipids formation after cell culture in adipogenic medium, Oil red staining was used. While cells grown on VO_x are visibly more marked comparing to the other samples, distinctive features of adipogenic differentiation, such as presence of spherical lipid vesicles, were not observed in large amounts (Fig. 3.25). As in the case of osteogenic differentiation described above, it can be explained by cell culture time period being less than the required to see clear signs of adipogenesis .

While Alizarin red, Alcian blue and Oil red stainings provided preliminary results on the differentiation behavior of hBMMSCs, it would be premature to make some conclusions based on observations described above. Most of the issues that we encountered analyzing obtained results could be solved by longer time periods of cell culture. Furthermore, reproducing cultures more times would ensure eliminating variations that originate from difference in contrast images etc.

In order to supplement the data on MSCs differentiation on TiO₂, Al₂O₃, VO_x thin films, we used a real-time PCR technique, the results of which are presented in the section 3.3.2

below.

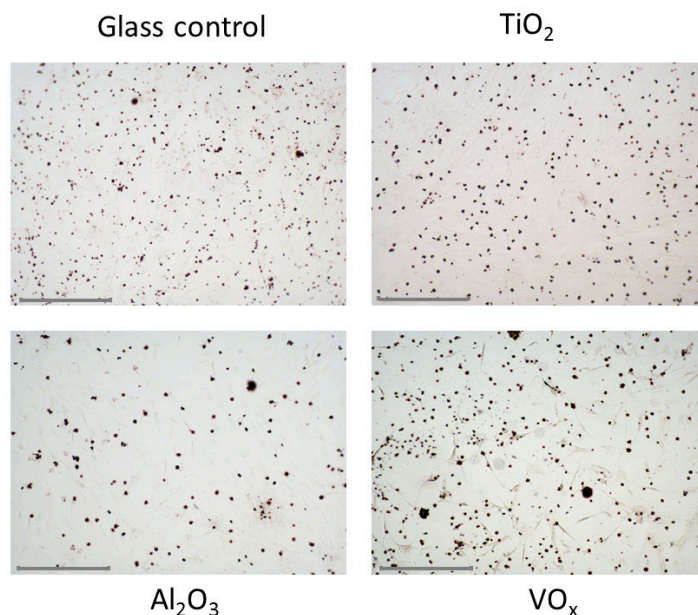


Figure 3.25. Oil red staining of MSCs seeded on glass control, TiO_2 , Al_2O_3 and VO_x -coated substrates after 14 days of cell culture. Scale bar = 500 μm .

3.3.2 Real-time PCR

For real-time PCR analysis, after cell culture for two weeks in standard α -MEM and appropriate differentiation media, OMD and SPARC genes were tested for osteogenesis, i.e. bone formation, COL2, ACAN and COMP genes were tested in terms of chondrogenesis, i.e. cartilage formation. In order to get complementary data about MSCs differentiation, expression of ELN and COL1 was analyzed as well. In each case comparison between experiment in standard and osteogenic or chondrogenic medium is provided. Although the process of particular stem cell differentiation is not expected with culture in standard medium, it was interesting to check whether there were signs of its initiation. Additionally, we evaluated expression of two transcription factors, RUNX2 and SOX9, which play a big role in regulation of osteogenic and chondrogenic differentiation, respectively.

To evaluate influence of oxide thin films on osteogenic differentiation of MSCs, we analyzed data on osteogenic markers which are presented in Fig. 3.26. It can be seen that

after cell culture with standard medium more RUNX2 was expressed on TiO_2 and Al_2O_3 thin films comparing to VO_x and glass control. However, in the case of culture in osteogenic medium the difference in expression between samples was leveled.

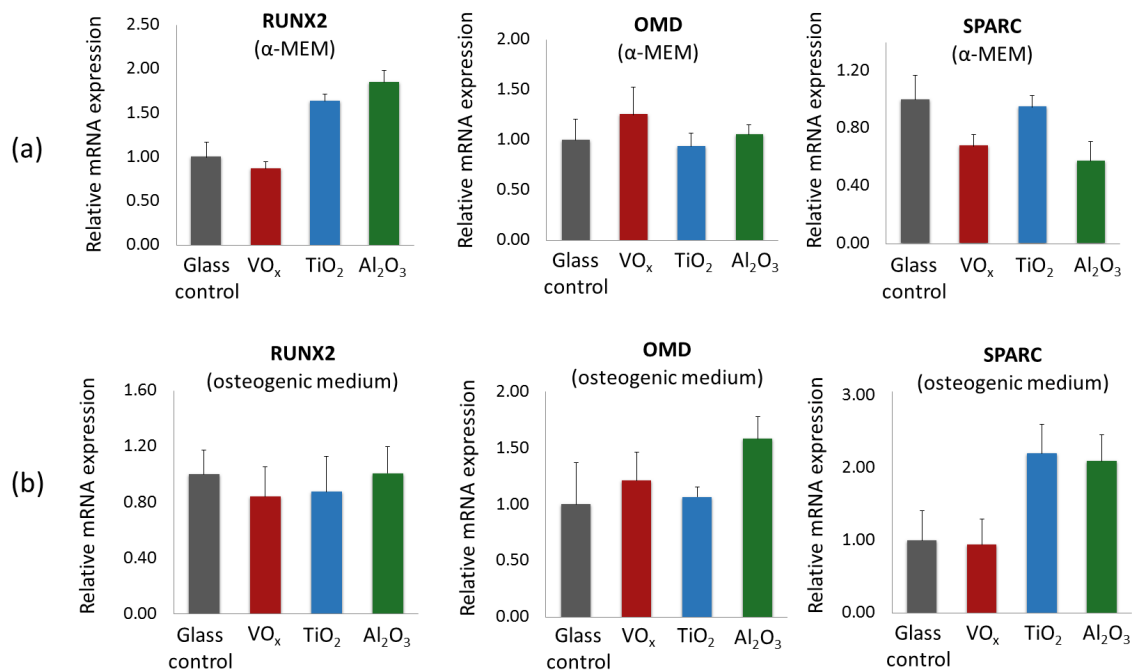


Figure 3.26. Osteogenic differentiation of hBMMSCs on the various samples and real-time PCR analysis of osteogenesis-related genes after culturing for 14 days. Relative expression of RUNX2, OMD and SPARC after cell culture in standard α -MEM medium (a) and osteogenic medium (b).

The statistical evaluation is performed by comparing the samples with the glass substrate.

While no significant difference in expression levels between VO_x and TiO_2 samples was noticed for osteomodulin (OMD), its elevated levels in comparison to glass control can be noted for Al_2O_3 . For another important osteogenesis-related gene, namely SPARC, up-regulation can be observed for cells seeded on TiO_2 and Al_2O_3 in differentiation media. Interestingly, in the case of culture in standard medium gene expression for Al_2O_3 samples, in contrary, was lower in comparison to glass control. There are few reports about effect of TiO_2 and Al_2O_3 on directing MSCs towards osteogenesis [52–56]. However, only one of them deals with using an oxide in the form of coating. To be more precise, Weng et al. employed thick double-layered Al_2O_3 coating with a porous outer layer to showcase its potential to

promote bone growth both *in vitro* and *in vivo* [55].

Concerning chondrogenic differentiation of MSCs, altered levels of COL1 and some chondrogenic markers, such as COMP and SOX9, can be observed in Fig. 3.27. It is worth noting that expression of ELN was also assessed, though the data could not be properly analyzed because of the melt curve peak doubling. Presence of multiple peaks in melt curves can be associated with contaminating amplification products or products not related to the target gene. However, such observations can be also explained simply by insufficient time of cell culture in osteogenic medium.

Data that was obtained on chondrogenic transcription factor SOX9 for cells grown on glass control and oxide thin films in standard and chondrogenic medium is presented in Fig. 3.27 (left). It can be seen that in comparison to other samples, Al_2O_3 enhances SOX9 expression after cell culture in standard medium. However, for cells grown in chondrogenic medium, SOX9 expression on Al_2O_3 and VO_x showed reduced levels in comparison to cells on glass control. Interestingly, significant down-regulation in the case of VO_x and Al_2O_3 can be also observed for expression of COL1. The expression of this gene is substantially related to the fibrocartilage, which formation is undesired during therapies aimed at restoring cartilage defects [57]. Therefore, such data can be interesting in terms of directing stem cells to differentiate into particular types of cartilage upon engineering necessary tissue.

It is worth to note that prior to running real-time PCR with target genes mentioned above, there were a number of tests with COL2 and ACAN genes, which are primary markers of chondrogenesis. Expression of these genes were detected only partly during these tests and it indicates the early stage of chondrogenesis as these genes are expected to be seen in the later stage. Despite the fact that expression of ACAN could not be observed properly, the altered levels for another gene related to the non-collagenous matrix, namely COMP, were detected. Elevated levels of this gene expression can be noticed for cells seeded on TiO_2 and Al_2O_3 thin films.

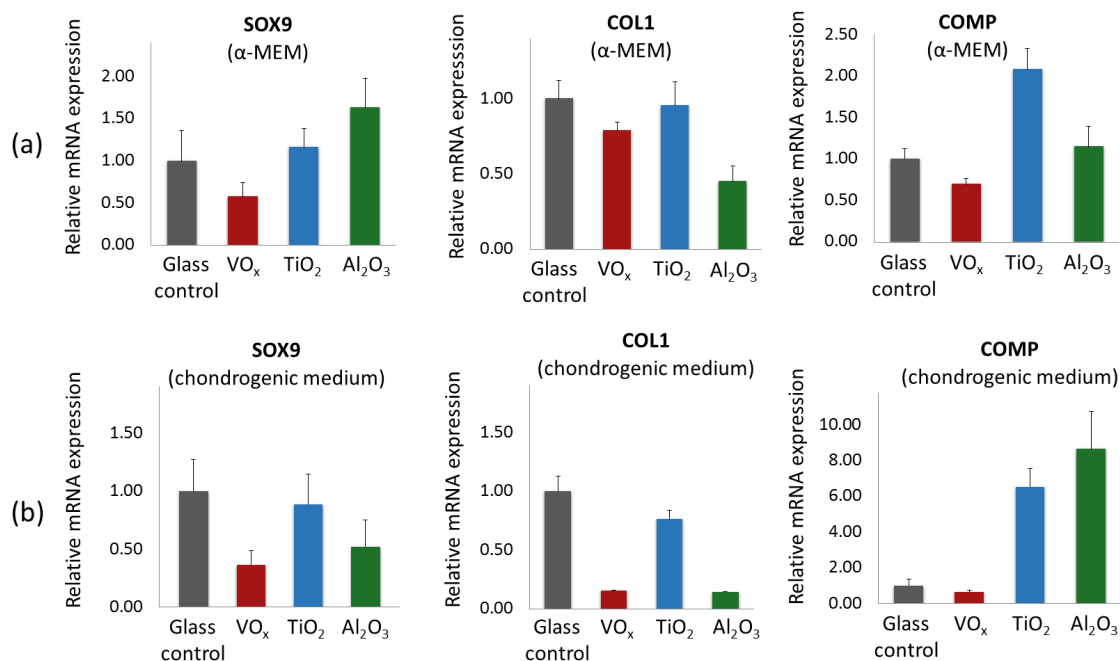


Figure 3.27. Chondrogenic differentiation of hBMMSCs on the various samples and real-time PCR analysis chondrogenesis-related genes after culturing for 14 days. Relative expression of SOX9, COL1 and COMP after cell culture in standard α -MEM medium (a) and chondrogenic medium (b). The statistical evaluation is performed by comparing the samples with the glass substrate.

Additionally, we made a preliminary assessment of expression of genes related to integrins, ITGA1 (Integrin subunit Alpha 1) and ITGB1 (Integrin subunit Beta 1). As described in the subchapter 1.3.2, integrins are cell membrane receptors having heterodimeric structure that composed of two subunits: alpha and beta. They are involved in mediation of the cell-ECM (extracellular matrix) interaction and cell-cell adhesion. It has been demonstrated that integrins also play a role in the differentiation of mesenchymal stem cells [58–60]. For example, LaPointe et al. examined the role of integrin $\beta 8$ in chondrogenic differentiation of hMSCs [59]. Results obtained within our study indicated remarkable difference in ITGA1 and ITGB1 expression between oxide thin films and control sample after cell culture in standard medium (Fig. 3.28a). To establish effect of oxide thin films on integrins expression further tests are required. However, in perspective, these data can serve as an input to the further planning of a series of experiments.

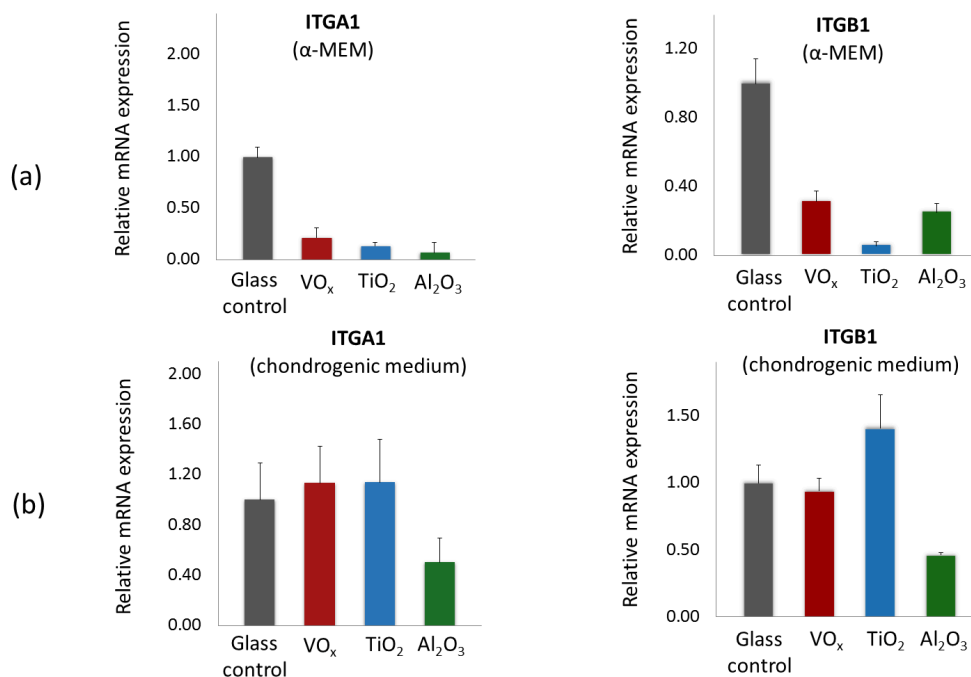


Figure 3.28. Comparison of relative expression of ITGA1 and ITGB1 after cell culture in standard α -MEM (a) and chondrogenic media (b). The statistical evaluation is performed by comparing the samples with the glass substrate.

3.3.3 Conclusion

In summary, the results of differentiation staining assays and real-time PCR analysis are in good accordance, both indicating potential of using oxide thin films to influence mesenchymal stem cell fate. The results on the effect of TiO_2 and Al_2O_3 thin films on osteogenic differentiation of MSCs obtained within this study are consistent with the data available in the literature. In addition to what was previously reported, we demonstrated that even smooth non-porous TiO_2 and Al_2O_3 thin films can not only support osteogenic potential of hBMMSCs, but also may play a role in inducing their osteoblastic differentiation. With respect to chondrogenic differentiation that was also assessed during our study, similar effect of TiO_2 was already reported [50]. However, Kim et al. studied TiO_2 in the form of nanotubes, while present study is focused on using oxides in the form of thin films. Furthermore, to the best of our knowledge, there were no studies on the potential impact of

Al_2O_3 and VO_x , in particular in the form of thin coatings, on chondrogenic differentiation of hBMMSCs.

Further studies would be valuable to see how oxide thin films affect cell differentiation path after cell cultures over the longer term. This, in turn, could be achieved by changing the amount of medium changes and reducing as well the value of plating cell density, since in our case time of cell culture was limited by the point where cells were reaching the confluency. Moreover, it has been demonstrated in other studies that initial cell density itself can be a factor influencing cell-material interaction and differentiation [61].

Overall, many methods and approaches can be used in order to direct stem cell to particular differentiation path. Surface chemistry and topography are the key parameters that can be effective for controlling stem cell differentiation [62–64]. It was demonstrated in a number of studies that varying substrate stiffness can be used to affect stem cell fate as well [65, 66]. Additionally, shape of a stem cell can also influence its lineage commitment [67]. As it was discussed in subchapter 3.1, there is a little difference in hydrophilicity of thin films that may affect cell spreading and eventually, along with difference in chemical composition of films surface, expression of various genes.

3.4 Influence of metal oxide thin films on the behavior of cancer cells

In order to further explore feasibility of using oxide thin films to study cellular-material interactions, we evaluated adhesion and proliferation of several cancer cell lines with ZnO, CuO, VO_x, Al₂O₃ and TiO₂ thin films deposited on the glass substrates. Uncoated glass substrate and plastic were used as controls. The latter, plastic well, is a common choice as substrate for cell cultures since it is specially pre-treated to facilitate cell attachment and growth.

3.4.1 Ovarian cancer cells

Two lines of ovarian cancer cells, IGROV1-R10 and SKOV3, were seeded on the surface of ZnO, CuO, VO_x thin films and two control samples, i.e. plastic and glass. Then, their adhesion and proliferation were followed by the means of IncuCyteTM live-cell analysis system. In Fig. 3.29 images of IGROV1-R10 cells on various substrates right after seeding and after 6, 24, 48 and 72 hours of culture are presented. As can be seen, there was no ostensive difference in morphology between cells growing on oxide coatings and control samples. Regarding the effect of oxides on adhesion and growth of IGROV1-R10 cells, thin films of VO_x exhibited a positive influence on cell attachment without interfering with their proliferation, which resulted in similar number of cells after 72 hours between control samples and VO_x. In turn, for CuO and ZnO thin films cytotoxic effect can be observed as there was visibly less cells starting from 24 hours of culture. While there is a limited number of studies on oxide thin films related to behavior of cancer cells, there have been numerous reports about cytotoxic effect of ZnO nanoparticles on cancer cells of various types [68–70].

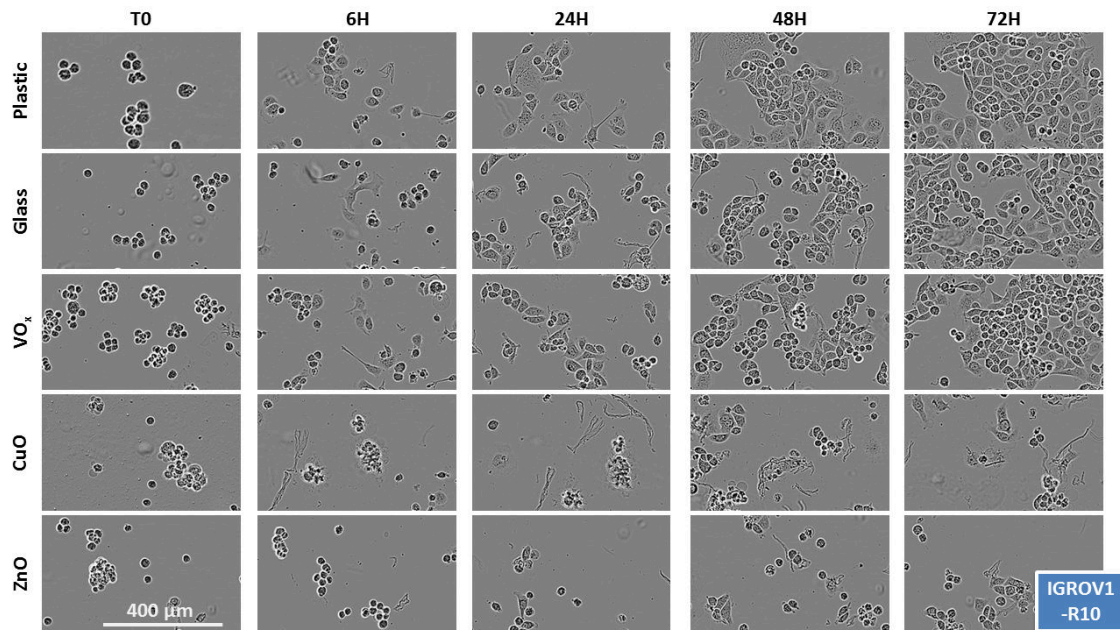


Figure 3.29. IGROV1-R10 on plastic, glass control, VO_x -, CuO- and ZnO-coated substrates.

The results of Cell Tox Green tests for IGROV1-R10 seeded on plastic, glass control, VO_x -, CuO- and ZnO-coated substrates indicate good coherence with live-cell imaging analysis. It can be seen in Fig. 3.30 that CuO and ZnO both show rates of cell apoptosis higher than control samples, while thin films of VO_x have comparable to plastic control rates.

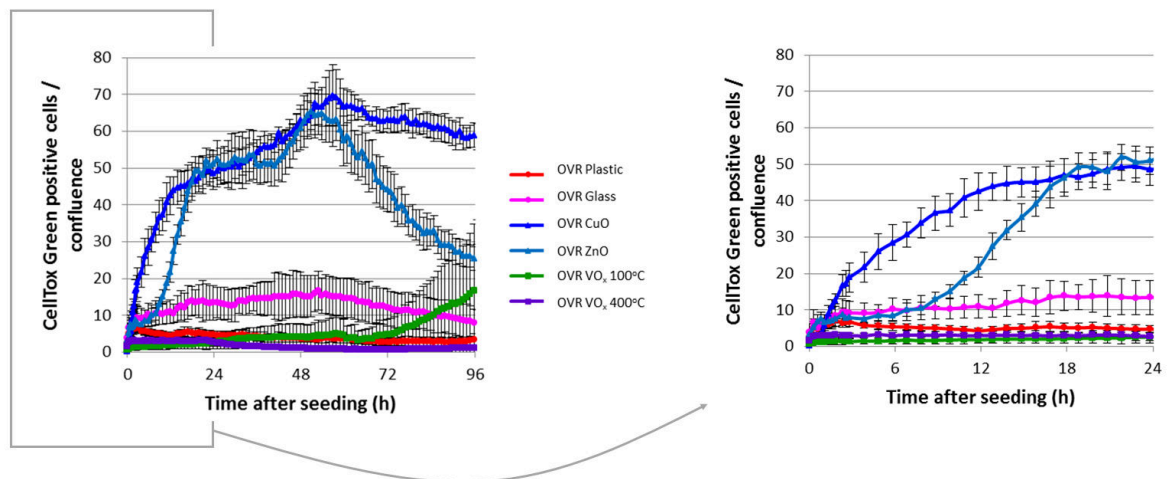


Figure 3.30. Cell Tox Green tests results for IGROV1-R10 seeded on plastic, glass control, VO_x -, CuO- and ZnO-coated substrates.

According to the analogous time-lapse microscopy images for SKOV3 (Fig. 3.31), cytotoxic effect was observed for ZnO thin films as distinctly fewer cells can be seen there in comparison to the other samples.

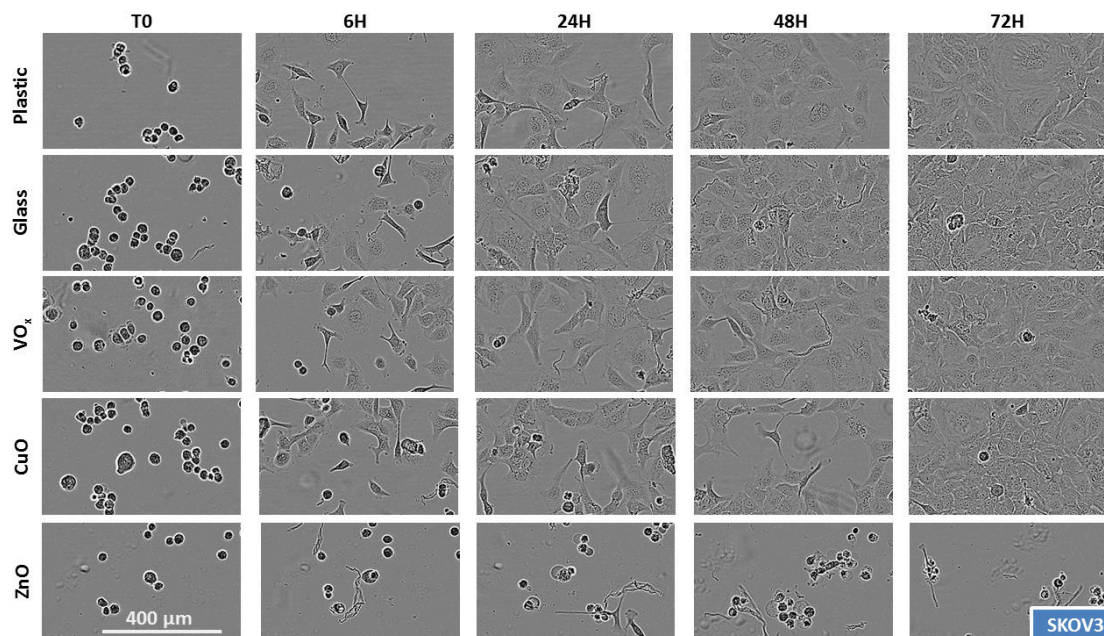


Figure 3.31. SKOV3 on plastic, glass control, VO_x -, CuO- and ZnO-coated substrates.

The data on the ratio between dead and viable cells acquired by Cell Tox Green assay also indicate higher cytotoxicity of ZnO towards SKVO3 cells (Fig. 3.32). Therefore, ZnO exert similar effect on the viability of both IGROV1-R10 and SKVO3 cell lines. Possible mechanism underlying in cancer cell death upon the contact with ZnO can be associated with altering of reactive oxygen species (ROS) level through oxidative stress [68]. Same assumption can be applied to explain the effect of CuO on IGROV1-R10 proliferation, since it has been already demonstrated that CuO nanoparticles can induce apoptosis of human cancer cells mediated by altering their oxidative stress [71, 72]. However, unlike ZnO, CuO thin films exhibit cytotoxic effect only towards SKOV3 and, in addition, such effect of metal oxides on ROS generation is possibly related exclusively to the presence of nanoscale particles in the materials.

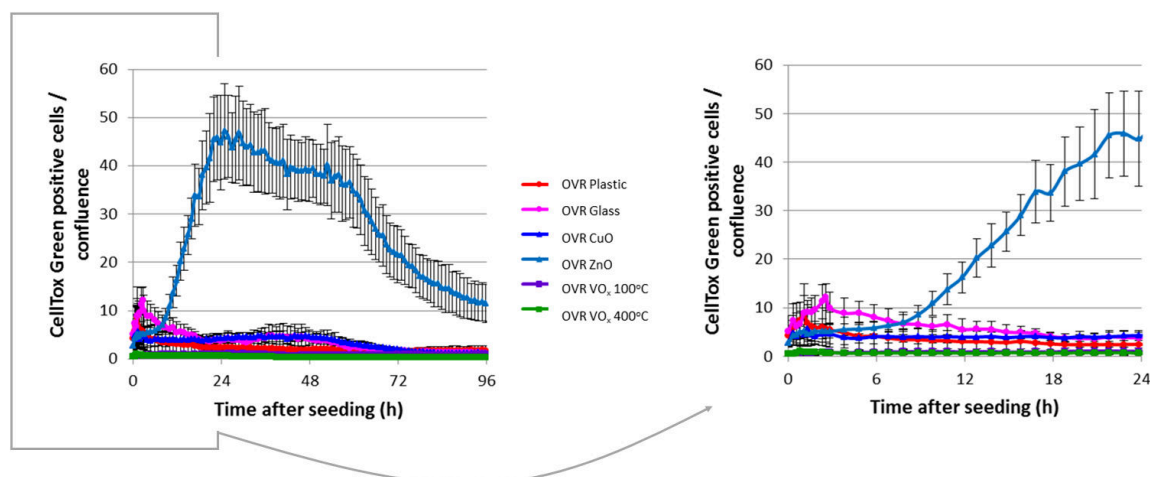


Figure 3.32. Cell Tox Green tests results for SKVO3 seeded on plastic, glass control, VO_x -, CuO- and ZnO-coated substrates.

3.4.2 Chondrosarcoma cells

As in the case of experiments on ovarian cancer cells, to evaluate the response of chondrosarcoma cancer cells to oxide thin films, different cell lines were used. In Fig. 3.33 - 3.36 images of JJ012, SW1353, FS090 and CH2879 cells onto plastic control and Al_2O_3 -, TiO_2 -, CuO-, ZnO- and VO_x -coated substrates are presented. The images were taken after 5 days of cell culture. For each particular sample, the amount and morphology of cells can be compared to the plastic control, which is a typically used substrate for cancer cell cultures.

In Fig. 3.33 it can be seen that while TiO_2 enhance proliferation of JJ012 cells, thin films of VO_x and especially CuO demonstrate an opposite effect. Concerning SW1353 cell line (Fig. 3.34), Al_2O_3 and TiO_2 thin films appear to support cell adhesion and growth on the same level as plastic control. For CuO, ZnO and VO_x , inhibition of SW1353 cells proliferation rate can be assumed as visibly fewer cells observed on the surface of these oxides after 5 days of cell culture.

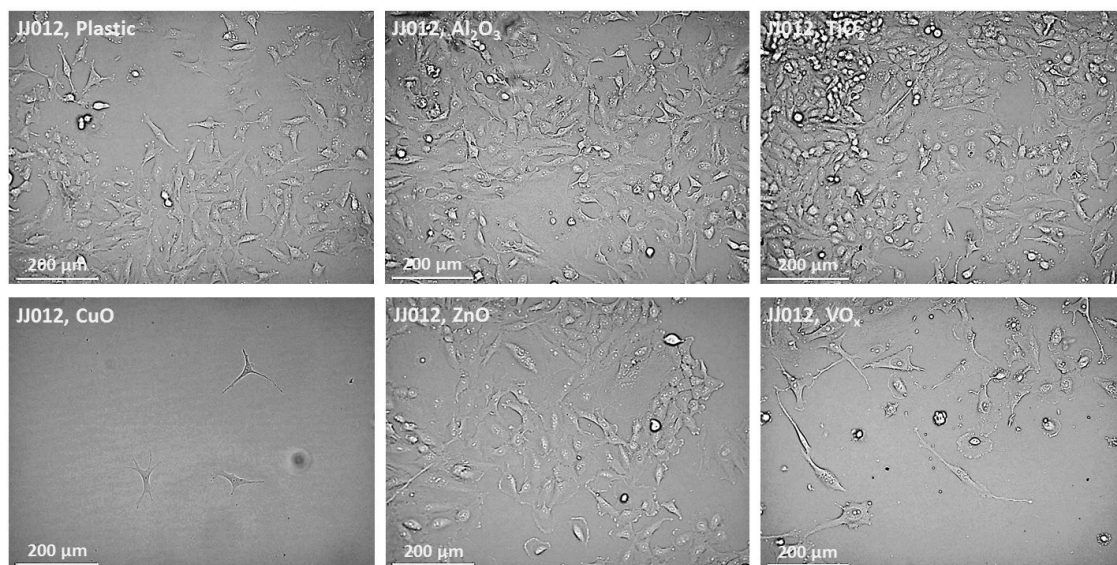


Figure 3.33. JJ012 cells on plastic, Al_2O_3 -, TiO_2 -, CuO -, ZnO - and VO_x -coated substrates (x20 magnification).

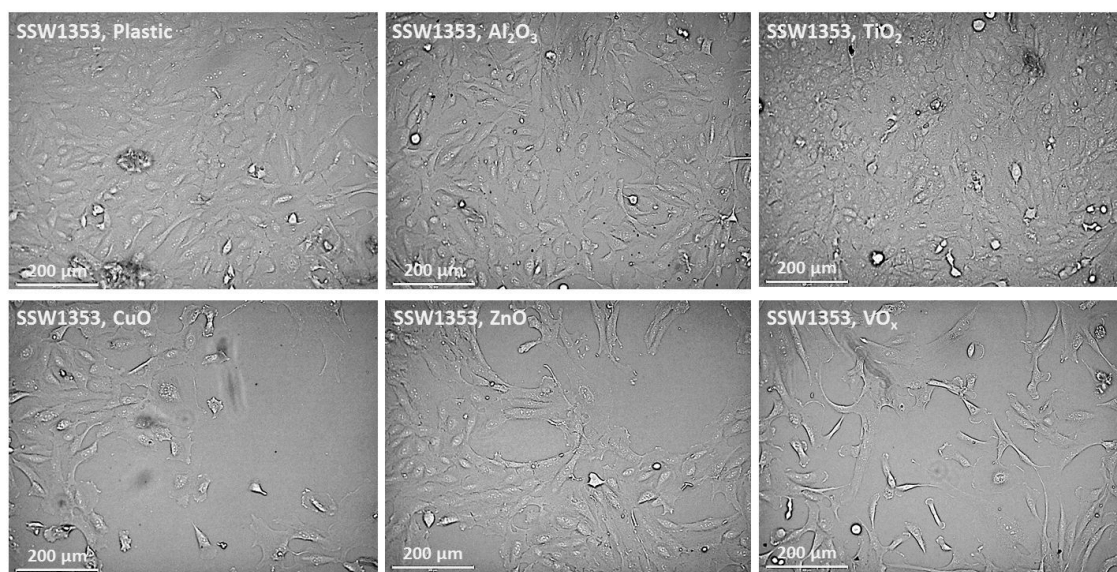


Figure 3.34. SSW1353 cells on plastic, Al_2O_3 -, TiO_2 -, CuO -, ZnO - and VO_x -coated substrates (x20 magnification).

Cells from another chondrosarcoma line, FS090, displayed a spindle-like shape on all of the substrates (Fig. 3.35), however, there was a little difference in the amount of cells, which

was more obvious in the case of TiO_2 (more cells) and CuO thin films (fewer cells). In Fig. 3.36 it can be seen that, apparently, all oxide thin films promoted higher rates of CH2879 cells proliferation in comparison to plastic control.

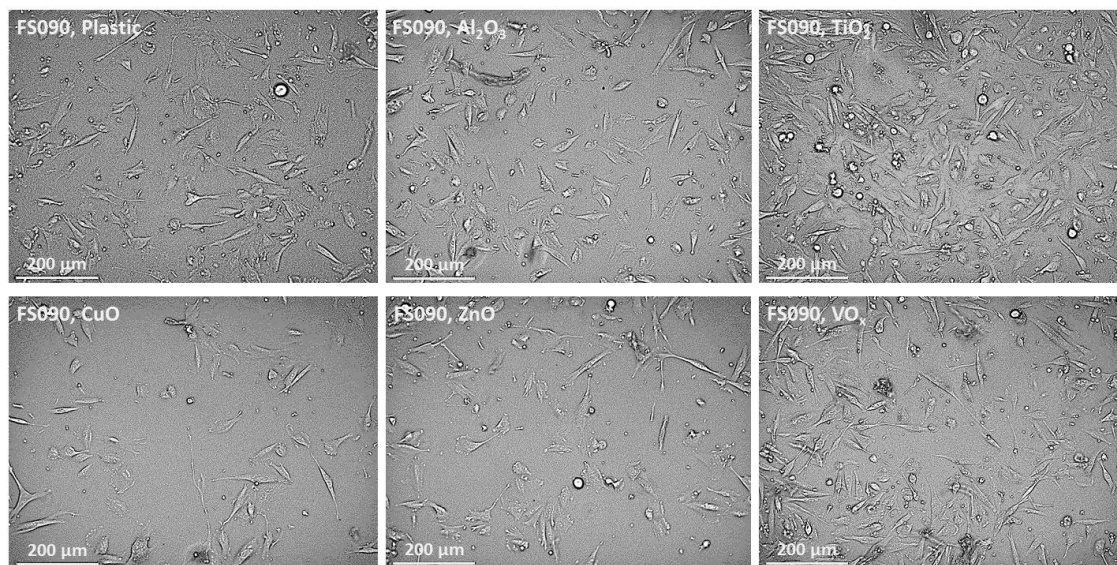


Figure 3.35. FS090 cells on plastic, Al_2O_3 -, TiO_2 -, CuO -, ZnO - and VO_x -coated substrates (x20 magnification).

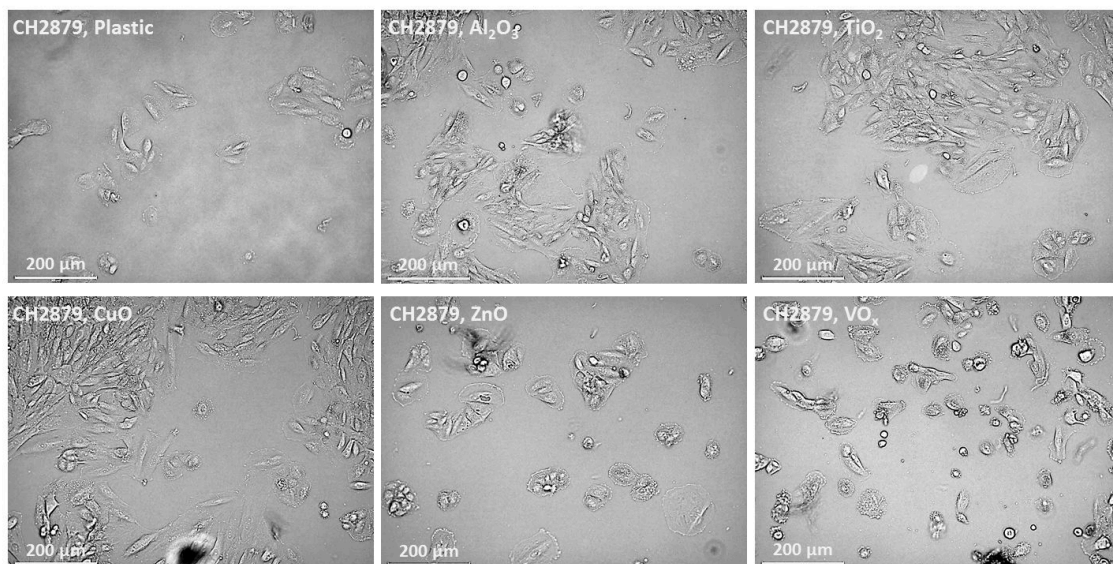


Figure 3.36. CH2879 cells on plastic, Al_2O_3 -, TiO_2 -, CuO -, ZnO - and VO_x -coated substrates (x20 magnification).

The difference between chondrosarcoma cell lines in the response to the same set of oxide films can be explained by the grade and classification of the tumor from which the cell lines were derived. In particular, CH cells are grade III and central chondrosarcoma, FS are grade II and myxoid, SW are grade II and central and JJ are grade II and both central and myxoid.

3.4.3 Conclusion

There are only a few works available with respect to using oxide thin films for interaction with cancer cells. In one of them MnO_2 nanospheres thin films were synthesized to develop an efficient substrate for capture and release of circulating tumor cells [73]. In another study, Dhayal et al. examined TiO_2 thin films deposited by magnetron sputtering system in terms of their effect on the growth of S180 sarcoma tumor cells [74].

For our experiments we chose cell lines that are associated with their resistance to the commonly applied treatment: IGROV1-R10 and SKOV3 are resistant to cisplatin used in chemotherapy, while chondrosarcoma cell lines are radioresistant [75, 76]. Results that we obtained indicate the potential of oxide thin films, such as VO_x , TiO_2 and some others, for the use in primary cultures. It would be interesting to investigate further whether these cells behave differently on thin films in comparison to their behavior in the tumor. The positive effect of some oxides on cell proliferation could be also interesting in terms of application in so-called organoid technology, which is associated with the formation of three-dimensional organoids mimicking characteristics of a tumor. Such approach currently gets a lot of attention, since it can provide a system to improve understanding of drug response and optimize cancer treatments [77, 78].

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4 Conclusions and perspectives

This study was concentrated on using thin films of metal oxides (TiO_2 , Al_2O_3 and VO_x) as substrates for human cells culture (bone marrow-derived mesenchymal stem cells, hBMM-SCs, and cancer cells). Oxide thin films were chosen as substrates since they can not only affect biocompatibility of the material and improve its mechanical properties, but also can serve as inhibitors of potentially harmful ion release. Very low values of surface roughness (0.1-0.2 nm) of thin films produced by Pulsed Laser Deposition (PLD) technique led to possibility of studying the influence of surface chemistry solely. This approach allowed following the relation between oxide thin films composition and corresponding cellular response.

In regards to the interaction of hBMMSCs with TiO_2 , Al_2O_3 and VO_x thin films, their adhesion, proliferation and differentiation was evaluated. First, we were able to show that TiO_2 and Al_2O_3 oxide thin films do not interfere with normal adhesion and proliferation. In turn, possible cytostatic effect of VO_x thin films could be observed. Second, by the means of real time Reverse transcriptase Polymerase Chain Reaction (RT-PCR) and staining assays, the effect of oxide thin films on differentiation path of mesenchymal stem cells was demonstrated. Although, further experiments are needed in order to explore these effects, the results revealed that oxide thin films can, indeed, affect the differentiation of MSCs. In particular, the results indicate the potential of using TiO_2 and Al_2O_3 thin films for directing MSCs towards osteogenic and chondrogenic differentiation pathways, which are relevant in terms of MSCs application in regenerative medicine and tissue engineering.

The difference in cell adhesion, proliferation and differentiation in the case of stem cells can be associated with the influence of surface chemistry and, consequently, its wettability. This surface phenomenon may be responsible for both variance in the protein adsorption and area of cell spreading, which in turn are among the parameters affecting cell behavior.

Another part of the present study was related to investigation of influence of oxide thin films on behavior of cancer cells. Two ovarian cancer cell lines (IGROV1-R10, SKVO3) and several metal oxides (ZnO , CuO , VO_x) in the form of thin coatings were utilized for these tests. The results of these experiments revealed positive effect of VO_x thin films on adhesion and proliferation of cancer cells, which is interesting in terms of using thin films of VO_x for

primary cell cultures. ZnO had a cytotoxic effect in case of both cell lines, namely IGROV1-R10 and SKVO3, while CuO showed cytotoxic activity only in case of IGROV1-R10 cells. Additionally, interaction of JJ012, SW1353, FS090 and CH2879 chondrosarcoma cell lines with Al₂O₃, ZnO, CuO, TiO₂ and VO_x thin film was evaluated. Significant differences in cell proliferation were observed for some of the combinations. The results for both ovarian cancer and chondrosarcoma cell lines reveal the potential of using oxide thin films for screening tests and investigation of cancer cells behavior.

Concerning the limitations of the present work, it should be noted that a longer periods of cell culture would allow to obtain more accurate data regarding differentiation staining and gene expression. Using substrates with a more appropriate size would also contribute to the accuracy of the results. Alternatively to the glass slides, titanium and its alloys, ceramics and many other conventional implant materials could be as well an interesting choice for cell culture substrates. In perspective, other types of cells (for example, bacteria) can be used for cultures. It would be also interesting to employ various surface modification methods, such as lithography and etching, in order to incorporate functional groups, change morphology and roughness of the surface.

In conclusion, cell behavior depends on a big number of factors and cell-material interaction can be very sensitive to variations of their environment and material properties. Therefore, it is often difficult to properly compare results of separate studies even if it concerns analogous chemical properties of the material. Such discrepancies can be minimized by studying as many compositions and cell types as possible within singular investigation and this can be done by using the approach similar to the one described in our work. We believe that obtained results provide a valuable addition to the scope of knowledge regarding influence of oxide thin films on cellular behavior.

1 Introduction et Revue de Littérature

1.1 Introduction

Au cours des dernières décennies, en particulier l'amélioration de la qualité et de l'espérance de vie est enjeu important. L'un des moyens d'y parvenir est de développer des matériaux pour remplacer les organes et les tissus endommagés. Des dizaines de matériaux différents (polymères, céramiques et métaux) ont été utilisés pour créer des articulations, des organes et des tissus artificiels, notamment des tissus osseux et cutanés, des fibres nerveuses et des vaisseaux sanguins. La fabrication de biomatériaux est un domaine interdisciplinaire, qui utilise des réalisations de la physique, de la chimie, de la médecine, de l'électronique et d'autres disciplines, et la recherche de nouvelles approches pour améliorer les performances des biomatériaux est en cours.

Les résultats de nombreuses études ont démontré que les propriétés de surface des biomatériaux sont cruciales pour orienter la réponse de la matière biologique [1, 2]. L'une des méthodes courantes de modification de la surface consiste à déposer un film mince sur un substrat approprié. Les revêtements métallo-oxydés ont attiré l'attention sur le champ de fabrication de biomatériaux dont certains sont bioactifs [3, 4], mais ce domaine de recherche reste largement inexploré. La plupart des études sur les potentialités des films minces à base d'oxydes en tant que cibles de croissance cellulaire portaient sur des surfaces en oxyde de titane, rugueuses ou modelées [5]. Diverses techniques, telles que la pulvérisation magnétron ou le revêtement par immersion, ont ainsi été utilisées pour un dépôt de dioxyde de titane. Il a été démontré que les revêtements à l'oxyde de titane ont non seulement une activité antimicrobienne, mais peuvent également améliorer l'adhésion et la prolifération des cellules [6-8].

Les facteurs déterminants pour identifier le potentiel du matériau comprennent sa stabilité thermodynamique, sa résistance à la corrosion et, bien sûr, sa non-toxicité et le fait

qu'il ne provoque pas de réactions allergiques. L'état actuel des connaissances suggère que la topographie de surface est le paramètre déterminant du comportement cellulaire et de la différenciation grâce aux interactions avec les protéines d'adhésion de la surface cellulaire, telles que les intégrines, qui modulent l'adhésion cellulaire, l'expression des gènes et le comportement cellulaire global [9]. La structuration importante pour le destin de la cellule se situe à l'échelle nanométrique, avec des éléments du même ordre de taille que le récepteur de la cellule. La plupart des études sur la croissance cellulaire sur films minces oxydés ont porté sur des surfaces modèles préparées par des techniques de fabrication micro/nano microélectroniques descendantes standard (surfaces typiques recouvertes de piliers, avec des variations systématiques de taille et des dimensions). D'une part, les inconvénients sont le coût élevé de ces techniques, la difficulté de passer à une production importante et l'optimisation pour un nombre limité de matériaux, et d'autre part, des surfaces résultantes manquent d'irrégularités qui semblent bénéfiques à l'adhérence cellulaire. Alternativement, il est possible de produire des surfaces désordonnées avec micro/nanotopographie par des techniques de surface changeantes (polissage, gravure, placage, anodisation, etc.), mais, bien que l'adhérence des cellules est améliorée, les résultats sont difficiles à comprendre en raison du manque de reproductibilité. Pour améliorer ces méthodes d'ingénierie, le développement de nouveaux biomatériaux fonctionnels est clairement nécessaire.

Les films minces d'oxydes, qui sont généralement utilisés pour leurs propriétés électroniques [10], constituent une option intéressante, car le contrôle et l'analyse de la surface sont simplifiés. Cependant, un nombre très limité d'oxydes a été étudié. Outre le TiO_2 , quelques études ont été effectuées sur le ZnO , le ZrO_2 et quelques autres oxydes. De plus, les mécanismes de l'interaction cellule-matériau ne sont pas encore complètement clairs. Ainsi, l'idée du présent travail est de minimiser l'influence de divers paramètres sur la réponse cellulaire sauf la chimie de surface, ce qui peut être obtenu grâce aux très faibles valeurs de rugosité de surface (0,1 à 0,2 nm) des couches minces produites par la méthode de l'ablation laser pulsé dite PLD (Pulsed Laser Deposition).

Les principaux objectifs de cette étude peuvent être résumés comme suit:

- identifier les couches minces d'oxydes métalliques biocompatibles,
- évaluer l'adhésion et la prolifération des cellules,
- étudier l'effet de la composition et d'autres paramètres sur la différenciation cellulaire.

Dans ce travail, des films minces de TiO_2 , Al_2O_3 , VO_x et quelques autres oxydes sont préparés et déposés sur des substrats de verre en utilisant un procédé de dépôt par ablation laser pulsé. Des cellules souches mésenchymateuses humaines dérivées de la moelle osseuse ont été cultivées à la surface des films minces et leur adhésion, leur croissance et leur différenciation ont ensuite été évaluées. De plus, l'adhérence et la prolifération de cellules cancéreuses ovariennes et de chondrosarcomes ont été également étudiées. Nos résultats illustrent le fait que les films minces à base d'oxydes métalliques, qui présentent une grande diversité de compositions et de propriétés physiques intrinsèques, constituent un choix très intéressant en tant que substrats pour les cultures de cellules dans les recherches portant sur l'interaction cellule-matériau. Nous pensons que cette étude démontrera la possibilité d'utiliser des films minces à base d'oxydes métalliques pour étudier le comportement de divers types de cellules.

1.2 Les films minces comme revêtements bioactifs

Avec les progrès technologiques rapides et les efforts en cours pour améliorer la qualité et l'espérance de vie, ainsi que les traitements cliniques existants, il existe une demande croissante pour la fabrication et la recherche de nouveaux biomatériaux. L'un des concepts essentiels en ce qui concerne les biomatériaux est leur biocompatibilité. Les matériaux biocompatibles sont définis comme des matériaux capables de s'intégrer dans le corps du patient (hôte) en produisant une réponse appropriée à partir du tissu biologique [11]. Parmi les domaines d'application des biomatériaux, il en existe trois principaux, à savoir: les implants orthopédiques, les matériaux de restauration dentaire, les implants cardiovasculaires, [12-14].

Selon les exigences réglementaires, un biomatériau doit posséder plusieurs propriétés importantes. Les biomatériaux ne doivent causer aucun effet allergique, toxique ou cancérigène sur le corps, ni provoquer de réaction inflammatoire ou d'infection locale. Conserver leurs propriétés fonctionnelles pendant la période d'exploitation prévue est une autre propriété que chaque biomatériau est censé posséder [15]. Les biomatériaux actuellement utilisés ne répondent pas toujours à toutes les exigences requises. Par exemple, les matériaux bioinertes avec une résistance mécanique suffisante ont souvent une faible intégration dans les tissus. À leur tour, les matériaux à haute bioactivité et compatibilité peuvent avoir de mauvaises propriétés mécaniques. Ce problème conduit à la recherche de nouvelles approches et de nouveaux matériaux pour les revêtements.

Le succès de l'application du matériau bioactif dépend fortement de la qualité de son intégration dans le tissu hôte. À son tour, l'intégration du matériau est directement liée à ses propriétés de surface, car la matière biologique au contact interagit immédiatement avec les couches de matériau les plus hautes. Les processus biologiques essentiels tels que l'adsorption de protéines et l'adhésion de cellules sont également des phénomènes de surface. Par conséquent, la modification évidente des propriétés chimiques et/ou chimiques de surface est le moyen simple d'influencer sur les performances du biomatériau. L'un des moyens les plus courants de modifier les caractéristiques de la surface, et sa réponse biologique consiste à déposer un film mince.

Outre leur capacité à améliorer la biocompatibilité du matériau, les revêtements de films minces présentent un grand intérêt car ils peuvent servir d'inhibiteurs de la libération potentielle d'ions nocifs, tout en améliorant de manière significative la stabilité mécanique et la résistance du matériau. De plus, les films minces sont largement étudiés en tant que revêtements dans la recherche sur la biologie des cellules souches et le développement de nouvelles approches pour les thérapies à base de cellules. Par exemple, les films minces peuvent être utilisés comme plates-formes efficaces pour la croissance et la différenciation des cellules souches, l'ingénierie des surfaces cellulaires et d'autres applications [16, 17].

Les éléments métalliques sont abondants et peuvent former des oxydes stables d'une

grande diversité de composition et de propriétés. Comme mentionné ci-dessus, de nombreux oxydes métalliques ont montré des résultats prometteurs dans le domaine des études biologiques. Une grande partie de ces études se concentre sur l'utilisation d'oxydes métalliques sous forme de nanoparticules. L'activité antibactérienne de nanoparticules de ZnO a été démontrée dans plusieurs études [34, 35]. Par exemple, Wahab et al. ont démontré l'effet positif des nanoparticules de ZnO sur la réduction de la prolifération des bactéries [35]. Ayant leurs avantages distinctifs, les nanoparticules sont néanmoins plus compliquées à caractériser, et à reproduire leurs propriétés de surface que les films minces.

L'une des premières applications de films minces à base d'oxydes qui a retenu l'attention des chercheurs a été l'amélioration des matériaux d'implant prothétique. Par exemple, les implants en titane (Ti) et leurs alliages sont largement utilisés en raison de leur biocompatibilité et de leurs bonnes propriétés mécaniques. Afin de minimiser les risques de défaillance des implants, il est important d'éviter la formation de biofilms bactériens à leur surface et d'assurer une interaction biologique appropriée entre l'implant et les tissus environnants. L'adsorption d'atomes d'oxygène de l'air entraîne la formation spontanée de la couche d'oxyde passif à la surface des matériaux au Ti. En cas de remplacement osseux, la nature bio-inerte de cette couche empêche un bon contact entre le tissu vivant et l'implant, entraînant ainsi une diminution de l'ostéointégration. De plus, la formation de la niche autour de l'implant augmente la probabilité d'adhérence bactérienne et d'inflammation. Outre les revêtements d'hydroxyapatite mentionnés ci-dessus, le dépôt de couches minces d'oxydes métalliques a été l'une des stratégies permettant de surmonter ces problèmes.

Par exemple, les films minces de TiO_2 ont beaucoup attiré l'attention en raison de leur activité photocatalytique. Les espèces réactives à l'oxygène, formées lors de l'irradiation UV, empêchent la colonisation bactérienne et renforcent l'ostéointégration de l'implant à base de Ti [36]. De nombreuses études ont démontré la bioactivité du TiO_2 , et ont indiqué que ces revêtements améliorent non seulement l'activité antimicrobienne du matériau, mais également sa stabilité chimique [37-44]. Les films minces de TiO_2 sont également considérés comme ostéoconducteurs et présentent une adhérence et une prolifération ostéoblastiques

supérieures par rapport aux substrats non revêtus [45, 46].

D'autres couches minces d'oxydes activement étudiées sont CuO, ZnO et ZrO₂. Ils sont connus pour leurs propriétés antibactériennes et les films minces de ces oxydes ont montré des résultats prometteurs pour la prévention de la formation de biofilms [47-50]. Les dépôts de revêtements de ZnO et de ZrO₂ ont également été utilisés pour étudier la biocompatibilité et l'activité ostéogénique de ces oxydes [51-53]. Par exemple, Trino et al. dans [51] ont conclu que les films minces de ZnO fonctionnalisés présentent des propriétés favorables à l'ostéointégration et aux caractéristiques mécaniques des implants. De plus, l'activité antibactérienne et l'impact sur le comportement cellulaire des films minces de Al₂O₃ ont également été évalués dans plusieurs études où les résultats ont montré le potentiel de cet oxyde dans des applications biomédicales, telles que la thérapie cellulaire et l'orthopédie [54-56].

En résumé, compte tenu de la diversité des compositions de films d'oxydes, le nombre d'oxydes métalliques étudiés en terme d'activité biologique est plutôt limité. En conséquence, cela permet un espace pour de nombreux travaux visant à améliorer les biomatériaux et les technologies biomédicales existants.

1.3 Les cellules souches mésenchymateuses

Les cellules souches mésenchymateuses (CSM) sont des cellules progénitrices multipotentes qui possèdent des propriétés d'auto-renouvellement et la capacité de se différencier en divers types de cellules qui forment des tissus et des organes spécifiques dans le corps humain, notamment des fibroblastes, des chondrocytes, des ostéoblastes, des myoblastes et des adipocytes.

Dans les études sur les thérapies utilisant des cellules souches, il est crucial de contrôler la différenciation. Pour diverses applications cliniques, il est nécessaire d'orienter la différenciation cellulaire d'une certaine manière en fonction de la réparation du vaisseau sanguin, de la formation de cellules épithéliales, etc. La trajectoire de différenciation des cellules

souches peut être influencée par un grand nombre de facteurs, tels que la présence d’une protéine spécifique ou propriété physique particulière du substrat [68, 69]. Il a été démontré que le destin des cellules souches peut aussi bien être défini en limitant, et en contrôlant la forme de la cellule [70]. De plus, la différenciation cellulaire peut être induite par la chimie de surface [71].

En résumé, les cellules souches peuvent réagir à tout changement dans les propriétés de surface, par exemple la chimie ou la topographie. L’environnement définit le devenir des cellules souches, leur adhésion et leur propagation, leur capacité d’auto-renouvellement et d’autres processus importants.

Jusqu’à présent, le mécanisme de la différenciation des CSM n’est pas tout à fait clair. En conséquence, de nombreuses études visent à déterminer les facteurs clés influençant le chemin de différenciation cellulaire. Compte tenu de tout ce qui précède, il est donc important de continuer à explorer les mécanismes inhérents aux processus d’adhésion et de différenciation cellulaires, car ils ne sont pas complètement compris.

1.4 Etude du comportement des cellules cancéreuses

Parfois, les cellules deviennent cancéreuses lorsqu’il y a une série de mutations. Ces mutations se produisent dans divers gènes qui régulent la prolifération cellulaire et sont généralement soit héritées, soit plus souvent causées par des agents cancérogènes dans notre environnement. Donc, contrairement aux cellules normales, la multiplication et la division des cellules cancéreuses se font de manière non réglementée avec réplication de cellules anormales et endommagées. Une autre différence réside dans la capacité des cellules cancéreuses à pénétrer dans les tissus voisins, ainsi qu’à circuler et se répandre librement dans les zones isolées du corps [73].

Malgré les énormes progrès réalisés dans la recherche sur le cancer ces dernières années, la compréhension et la guérison de cette maladie doivent encore être améliorées. Dans cette étude, plusieurs lignées de cellules cancéreuses ont été utilisées pour des expériences

concernant leur interaction avec des films minces à base d'oxydes métalliques. L'une d'elles est des lignées cellulaires du cancer de l'ovaire, car le cancer de l'ovaire est considéré comme l'un des types de cancer les plus répandus chez les femmes et figure parmi les principales causes de décès [75]. Les cellules dérivées du chondrosarcome, une tumeur maligne composée principalement de cellules produisant du cartilage, sont un autre type de cellules cancéreuses utilisées [76].

1.5 Techniques de dépôt de couches minces

Au cours des dernières décennies, les films minces ont eu un impact considérable sur la variété des applications. Ils sont utilisés pour les dispositifs optiques et de stockage d'énergie, et dans des domaines tels que la nanolithographie, les revêtements de durcissement, la thermoélectricité et bien d'autres [77, 78]. Différentes méthodes sont utilisées pour synthétiser un film mince, chacune ayant ses avantages et ses inconvénients. Généralement, ils sont divisés en techniques de dépôt physique et chimique.

Le principe de base des méthodes chimiques est le dépôt de précurseurs en phase gazeuse ou liquide sur le substrat à la suite d'une réaction chimique.

Les méthodes physiques sont largement étudiées et certaines d'entre elles sont les méthodes les plus fréquemment utilisées pour le dépôt de films minces. Fondamentalement, elles reposent toutes sur quelques étapes de base, telles que le transport du matériau de la cible au substrat, suivi de sa condensation, formant ainsi le revêtement. Parmi les techniques de dépôt physique, on peut distinguer les méthodes d'évaporation et de pulvérisation cathodique. Toutes les techniques d'évaporation comprennent l'évaporation thermique d'atomes de la cible à l'état solide qui sont transférés au substrat dans la phase gazeuse ainsi formée. Tous ces processus sont effectués sous atmosphère contrôlée ou sous vide.

Ablation laser pulse (PLD). Cette technique de dépôt est largement utilisée pour produire et étudier des surfaces nouvelles. Le processus typique de PLD se déroule dans une chambre à vide et consiste en une ablation au laser du matériau cible placé devant le substrat. Le

faisceau laser pulsé à haute énergie est focalisé sur la cible à travers le système de lentilles et, lorsqu'il atteint la cible, il se produit un échauffement local rapide de la surface qui se traduit par la vaporisation de la cible et la formation d'un plasma. Le panache de plasma ainsi créé contient des ions, des électrons, des atomes, des molécules, des grappes et même des macroparticules fondues d'un matériau cible. Ces espèces se propagent ensuite vers le substrat sur lequel le revêtement est déposé [113].

Par conséquent, PLD fournit un outil polyvalent et puissant pour la synthèse de films minces pour une large gamme d'applications, comme les supraconducteurs, les oxydes complexes et d'autres matériaux fonctionnels [118-120]. Concernant les études de biomatériaux, il a été utilisé avec succès pour déposer des revêtements biocompatibles, notamment de l'hydroxyapatite (HA), qui présentent un grand intérêt pour les études de régénération osseuse grâce à leur ressemblance avec les os naturels [121, 122]. Par exemple, Rajesh et al. dans [122] ont utilisé la technique PLD pour déposer une couche d'hydroxyapatite stable et adhérente sur un substrat de titane avec une couche intermédiaire d'oxyde de titane.

Pour résumer, l'objectif de cette thèse est de montrer la faisabilité des couches minces d'oxydes métalliques déposées par PLD afin d'évaluer le comportement des MCS et des cellules cancéreuses dérivés de la moelle osseuse humaine lors de leur contact avec ces oxydes. Le système PLD pour la synthèse de couches minces permet d'introduire un flux d'O₂ dans la chambre pendant le processus de dépôt, ainsi que de modifier les paramètres de dépôt tels que la température, la pression à l'intérieur de la chambre à vide et le nombre et l'énergie des impulsions appliquées. Cela donne l'occasion d'étudier l'effet non seulement de la composition chimique de la cible d'oxyde oxydée, mais également de l'effet de tous ces paramètres de dépôt sur le comportement des cellules souches mésenchymateuses et des cellules cancéreuses.

2 Méthodologie

2.1 Préparation de films minces d'oxydes par ablation laser pulse

Des films minces ont été déposés sur des substrats de verre en utilisant la technique PLD. Les cibles TiO_2 , Al_2O_3 et VO_x sont préparées par une voie standard à l'état solide. Des lamelles de verre rondes ont été utilisées comme substrats ($\varnothing = 12 \text{ mm}$). Elles sont collées avec de la laque d'argent sur l'élément chauffant avec un masque circulaire. Le dépôt a été effectué en utilisant un laser à excimères KrF ($\lambda = 248 \text{ nm}$). Pour chaque échantillon, le nombre d'impulsions était de 5000 avec un taux de répétition de 5 Hz. Le dépôt est effectué à différentes températures allant de 100 à 500°C. Avant chaque dépôt, la chambre était évacuée avec une pompe turbo à une pression de base inférieure à $1.33 \times 10^{-4} \text{ Pa}$ (10^{-6} Torr). Au cours des expériences impliquant un dépôt avec O_2 , du gaz a été introduit dans la chambre avec un débit qui a conduit à une pression de $10^{-4} - 10^{-5} \text{ Torr}$.

2.2 Caractérisation des films minces deposees

Diffraction des rayons X. Dans cette étude, les diffractogrammes sont enregistrés à l'aide du diffractomètre BrukerTM D8 Discover avec rayonnement $\text{CuK}\alpha_1$ ($\lambda = 1,541 \text{ \AA}$) et une plage d'angle 2Θ de 10 à 50°.

Réflectivité aux rayons X. L'épaisseur des échantillons a été caractérisée à l'aide de mesures de réflectivité aux rayons X (XRR), dans lesquelles la position relative des maxima d'oscillation par rapport au carré de leur numéro d'ordre a été ajustée pour obtenir l'épaisseur du film à partir de la pente. Les courbes de réflectivité ont été enregistrées à l'aide du diffractomètre BrukerTM D8 Discover avec rayonnement $\text{CuK}\alpha_1$ ($\lambda = 1,541 \text{ \AA}$) et angle 2Θ compris entre 0 et 6°.

Microscopie à force atomique (AFM). Pour la présente étude, la rugosité moyenne quadra-

tique (valeur quadratique moyenne) a été calculée à partir de mesures AFM d'une surface de $1\ \mu\text{m}^2$ en mode taraudage et calculée à l'aide du logiciel WSxM 5.0.

Mesure de l'angle de contact. L'angle de contact des surfaces a été évalué par un système d'analyse de la forme d'une goutte (DSA, Kruss, Allemagne) par la technique de la goutte sessile ($1\ \mu\text{l}$). Pour chaque échantillon, l'angle de contact moyen final a été obtenu à partir d'au moins trois mesures.

2.3 Culture cellulaire

Cellules souches mésenchymateuses dérivées de la moelle osseuse humaine. La moelle osseuse a été obtenue à partir des crêtes iliaques de donneurs adultes subissant une arthroplastie et qui ont signé un formulaire d'accord conformément au comité d'éthique local. La moelle est aspirée et les cellules sont fractionnées sur un gradient de densité Hypaque-Ficoll. Les cellules mononucléées sont ensuite isolées et développées dans du milieu alpha-MEM additionné de 10% de sérum de veau fœtal (InvitrogenTM, Cergy-Pontoise, France), de L-glutamine 2 mM, de 1 ng/ml de FGF-2 (SigmaTM) et d'antibiotiques. Aux environs de 80% de la confluence, les cellules sont récoltées par trypsination (trypsine à 0,25% / EDTA 1 mM, InvitrogenTM) et ensemencées à 2×10^3 - 5×10^3 cellules/cm². Après 5 passages, les cellules sont contrôlées par RT-PCR pour l'absence de marqueurs hématopoïétiques. Pour les expériences in vitro, des CSMh sont ensemencés à une densité de 5×10^4 cellules/cm² dans une plaque à 24 puits. Le milieu de culture est changé 3 fois par semaine, et maintenu dans une atmosphère à 5% de CO₂ à 37°C.

Cellules cancéreuses de l'ovaire. Les lignées cellulaires de carcinome ovarien chimiorésistantes SKOV3 et IGROV1-R10 sont cultivées dans du milieu RPMI 1640 supplémenté avec 2 mM de Glutamax, 10% de sérum de veau fœtal, 20 mM HEPES et 33 mM de bicarbonate de sodium et sont maintenues dans une atmosphère humidifiée à 5% de CO₂ à 37°C.

Cellules de chondrosarcome. Les lignées cellulaires de chondrosarcome humain SW1353, FS090, CH2879 et JJ012 sont ensemencées à 10^4 cellules par puit dans des plaques à 24

puits dans du milieu DMEM supplémenté avec 2 mM de GlutamaxTM, 10% de sérum de veau fetal, 20 mM de HEPES et 33 mM de bicarbonate de sodium et sont conservées dans une atmosphère humidifiée à 5% de CO₂ à 37°C.

2.4 Etude de l'adhésion et de la prolifération cellulaire

Microscopie à contraste de phase. L'adhérence et la prolifération des cellules ont été contrôlées au moyen d'un microscope VisiScopeTM série 400 (VWR) à contraste de phase et d'un système d'imagerie EVOSTM FL Auto 2. Pour suivre l'évaluation du diamètre des structures multicellulaires, des CSMh ont étéensemencés à une densité de 10⁴ cellules/cm² dans une plaque de 24 puits contenant des films minces de TiO₂ et Al₂O₃, ou un substrat verre servant de témoin. Les milieux sont changés 3 fois par semaine et maintenus dans une atmosphère à 5% de CO₂ à 37°C pendant 26 jours. Pour chaque échantillon, des structures multicellulaires sont observées sous microscope VisiScopeTM série 400 (VWR) à contraste de phase.

Test de viabilité et de prolifération cellulaire WST-1. Les WST (sels de tétrazolium hydrosolubles) appartiennent à la famille des colorants et le principe du test WST-1 est basé sur la détection de cellules viables lorsque ce colorant est réduit à l'extérieur de la cellule. La réduction conduit à un changement de couleur ultérieur, qui a été détecté par des mesures de densité optique avec un spectrophotomètre Multiskan GO. L'absorbance était enregistrée à une longueur d'onde égale à 450 et 600 nm. Ce test est utilisé uniquement pour comparer la cytotoxicité des couches minces de VO_x déposées à des pressions normales et partielles d'oxygène avec le contrôle du verre.

Coloration histologique. Pour la coloration histologique, des CSMh sontensemencés à une densité de 10⁴ cellules/cm² dans une plaque à 24 puits sur différents substrats, y compris des lames de verre non revêtues, ainsi que des lames à films minces en oxyde. Les milieux sont changés 3 fois par semaine et maintenus dans une atmosphère à 5% de CO₂ à 37°C pendant 14 jours. Après, les cellules sont rincées deux fois avec du PBS et fixées avec du

paraformaldéhyde à 4% pendant 15 minutes avant un autre rinçage avec du PBS, puis les cellules sont maintenues dans ce tampon jusqu'à une analyse plus poussée. Pour chaque échantillon, les résultats de Van Gieson, d'hématoxyline phloxine safran et de trichrome de Massons ont été fixés sur des lames de verre histologiques microscopiques avec une solution de polymère acrylique et observés avec le système d'imagerie EVOSTM FL Auto 2.

La microscopie électronique à balayage. La morphologie des CSM cultivées sur des couches minces d'oxydes est étudiée avec un microscope électronique à balayage (SEM) ZeissTM SUPRA 55 fonctionnant dans la plage de tensions comprises entre 2.0 et 10.0 kV, et une distance de 4 à 10 mm. Dans notre étude, des CSMh cultivés pendant 2 semaines ont été fixés avec 2,5% de glutaraldéhyde dans du tampon cacodylate 0.1 M (pH = 7.4) à 4°C, rincés avec du tampon cacodylate et postfixés dans du tétroxyde d'osmium à 1%. Ensuite, ils sont déshydratés progressivement avec de l'éthanol (70 - 100%) et séchés dans un séchoir à point critique (CPD 030 LEICA Microsystem). Les échantillons sont pulvérisés avec du platine (Pt), et observés à l'aide d'un microscope électronique à balayage SUPRA55 (ZeissTM).

Mesures de densité cellulaire. Pour les mesures de croissance de la population de cellules souches mésenchymateuses, des CSMh sontensemencés à une densité de 10^4 cellules/cm² dans une plaque à 24 puits contenant des films minces de TiO₂ et Al₂O₃, ou un substrat de verre comme témoin. Les milieux sont changés quotidiennement pour le milieu frais pendant cinq jours. Les cellules sont récoltées et numérotées séparément en utilisant une chambre de numération Malassez.

Plus tard dans l'étude, pour les expériences concernant l'utilisation de la même température de dépôt pour des couches minces d'oxydes, des CSMh sontensemencés avec du milieu α -MEM à la densité de 10^4 cellules/cm² dans une plaque à 24 puits sur différents substrats, y compris des couches minces de TiO₂, VO_x et Al₂O₃ ou lame de verre non revêtue. Au bout de deux heures, les milieux sont retirés et les cellules sont lavées avec une solution saline tamponnée au phosphate pour éliminer les cellules non adhérentes. Les CSMh sont ensuite récoltées par trypsination (trypsine à 0,25% / EDTA 1 mM, InvitrogenTM) et numérotés séparément à l'aide d'un compteur de cellules automatisé coloré au bleu trypan

et CountessTM II (Thermo Fisher Scientific). La même procédure est réalisée pour compter les cellules après la mise en culture à différents moments (2, 4 et 7 jours).

2.5 Etude de la différenciation des cellules souches mésenchymateuses humaines

Coloration de différenciation. Afin d'évaluer l'influence des films minces d'oxydes sur la capacité des CSM à se différencier en différents types de cellules, une coloration de différenciation est réalisée. Pour cela, les CSM sont cultivées avec un milieu α -MEM standard (sans facteur de croissance de fibroblaste basique) pendant 7 jours. Ensuite, les milieux de différenciation chondrogénique, ostéogène et adipogène sont ajoutés pour les colorations au Alcian Blue, Alizarin Red et Oil Red, respectivement. Les médias sont changés tous les 2-3. Après 14 jours de culture au total, les cellules sont fixées avec du paraformaldéhyde (PFA) à 4% et, après avoir été lavées avec une solution saline tamponnée au phosphate (PBS), les cellules sont conservées dans 1,5 ml de PBS pour des tests de coloration différenciés supplémentaires. Pour fixer les échantillons après coloration sur des lames de verre histologiques microscopiques, une solution d'un polymère acrylique est utilisée.

RT-PCR en temps réel. La réaction en chaîne de la polymérase par transcription inverse en temps réel (RT-PCR en anglais) est une méthode largement utilisée pour mesurer l'expression génique. La culture cellulaire est réalisée en utilisant un milieu standard (α -MEM) et un milieu de différenciation. COL2, ACAN, COMP, SOX9, ELN et autres ont été testés pour la chondrogenèse, c'est-à-dire la formation de cartilage. OMD, RUNX2 et SPARC sont testés pour l'ostéogenèse, c'est-à-dire la formation osseuse. Le colorant SYBR Green est utilisé pour la détection de l'ADN double brin et les niveaux d'expression de l'ARNm sont calculés par la méthode $2^{-\Delta\Delta C_t}$.

3 Résultats et Discussion

3.1 Caractérisation des films minces déposés

Comme il a été mentionné dans le premier chapitre, l'interaction entre la surface et la matière biologique, par ex. cellules, dépend fortement d'un certain nombre de facteurs, tels que la composition chimique de la surface, sa morphologie et sa porosité, sa structure cristallographique, sa mouillabilité, etc. La réponse et le comportement cellulaires au contact du matériau peuvent être très sensibles aux modifications, même minimales, de ses propriétés physiques ou chimiques. Il est donc important de caractériser le matériau avant de l'utiliser pour des cultures cellulaires et des tests biologiques. Dans le cadre de cette étude, avant les expériences *in vitro*, diverses techniques ont été utilisées afin d'obtenir des données sur l'épaisseur, la rugosité, la mouillabilité et d'autres propriétés du film. Plus de 350 échantillons ont été utilisés pour la culture cellulaire et les résultats de l'analyse démontrent un bon contrôle et une bonne reproductibilité des propriétés des films minces déposés par ablation laser.

Diffraction des rayons X. Les oxydes de titane et d'aluminium peuvent subir des transitions de phase induites par la température ou la pression [1, 2]. Cependant, malgré toutes les phases possibles, en ce qui concerne l'état d'oxydation des métaux, ils sont généralement stables avec le rapport métal sur oxygène suivant: TiO_2 et Al_2O_3 (souvent appelé oxyde de titane et alumine). À savoir avec ces formules chimiques, ils existent dans la nature sous forme de minéraux, où le rutil et le corindon sont les plus abondants pour TiO_2 et Al_2O_3 , respectivement. Contrairement au titane et à l'alumine, le vanadium forme divers oxydes en fonction de la température et d'autres conditions, comme l'indique le diagramme de phases représenté à la Fig. 3.1a [3]. La diffraction des rayons X a été réalisée pour obtenir l'information sur le dépôt spécifique de l'oxyde de vanadium au cours de l'expérience. Les mesures DRX ont révélé que les films minces déposés avaient une structure amorphe. Les

diagrammes de diffraction pour les couches minces d'oxyde de vanadium déposées sur des substrats de verre à 300 et 500°C sont présentés à la Fig. 3.1b, où l'on peut voir deux larges pics. Ces pics de faible intensité apparaissent en cas d'absence d'ordre à longue portée dans la structure. Les données DRX indiquent que les températures élevées entraînent des modifications de la structure cristallographique, et que l'oxyde de vanadium déposé à 300°C présente une structure plus amorphe; les pics de diffraction apparaissant plus larges et d'intensité plus faible par rapport au film déposé à une température plus élevée. Néanmoins, l'analyse par diffraction des rayons X n'a pas permis d'avoir une certitude sur la stoechiométrie particulière de l'oxyde de vanadium et, par conséquent, la composition des films minces en oxyde de vanadium sera notée VO_x .

Réflectivité des rayons X. Les résultats de l'analyse XRR montrent que l'épaisseur des films d'oxydes déposés à la même température peut varier en fonction de la composition cible (Fig. 3.2a). Etant donné que la même énergie laser et le même taux de répétition ont été utilisés lors du dépôt des trois oxydes, la variance en épaisseur est associée à la différence de propriétés optiques des cibles solides en oxyde métallique. À leur tour, les propriétés optiques dépendent de la structure électronique intrinsèque de l'oxyde métallique particulier. Ainsi, cela conduit à une profondeur d'adsorption laser différente pour chaque oxyde et, par conséquent, à un taux d'ablation, qui correspond à la quantité de matériau transférée de la cible au substrat. En général, on constate qu'une augmentation de la température de dépôt entraîne des périodes d'oscillations plus courtes, c'est-à-dire des valeurs plus élevées de l'épaisseur du film (Fig. 3.2b), qui peut s'expliquer par l'effet de l'échauffement entraînant une augmentation de la mobilité des espèces éliminées.

Microscopie à force atomique. La comparaison des profils de surface du substrat de verre et des couches minces d'oxydes déposés à la même température (300°C) ne révèle pas non plus de grande altération de la rugosité de surface (Fig. 3.3). On constate que malgré la composition différente, tous les films d'oxyde déposés possèdent une surface nanostructurée uniforme avec des valeurs de rugosité moyenne inférieures à 1 nm. L'analyse des mesures AFM des couches minces déposées dans le cadre de cette étude révèle que tous les revêtements

ont une surface très lisse et plate avec une taille de grain moyenne inférieure à 100 nm. Les valeurs efficaces se situent entre 0.1 à 0.5 nm, ce qui indique que les éclaboussures ne sont pas significatives.

Mesure de l'angle de contact. Dans cette étude, les angles de contact se sont révélés respectivement égaux à 61.72, 70.02 et 85.30° pour les films de VO_x , TiO_2 et Al_2O_3 déposés à 300°C. Cela indique que la surface de l'oxyde d'aluminium est moins hydrophile que la surface de TiO_2 et de VO_x . Globalement, concernant les couches minces d'oxydes, aucune différence significative n'a été observée entre les diverses conditions de croissance, et la surface des couches analysées apparaît hydrophile avec un angle de contact de l'eau inférieur à 90 degrés (Fig. 3.4c-e).

La spectroscopie à rayons X à dispersion d'énergie. La figure 3.5 présente les spectres EDS de films minces de TiO_2 et Al_2O_3 déposés sur des lames de verre. Les résultats révèlent des pics forts à environ 0.5, 1.1 et 1.8 keV correspondant aux signaux des atomes d'oxygène, de sodium et de silicium, respectivement. Alors que les pics de Si et de Na proviennent uniquement du substrat de verre, les pics d'O proviennent à la fois du substrat et du film d'oxyde. En analysant les intensités relatives des pics Si et O et en les comparant entre elles, on peut constater que le rapport entre ces pics est différent pour TiO_2 et Al_2O_3 . Cette observation peut indiquer une distribution différente de l'atome d'oxygène à la surface des oxydes. Par conséquent, cela peut être l'une des raisons de la différence de comportement des cellules pendant qu'elles sontensemencées à la surface de TiO_2 et de Al_2O_3 .

Afin d'obtenir des informations plus fiables sur la distribution des atomes dans les films minces, un substrat ne contenant pas d'atomes de O, Ti et Al pourrait être utilisé. Cela exclurait la superposition des signaux provenant de couches minces de substrats et d'oxydes. Pour cette raison, une telle approche est préférentielle et utilisée dans de nombreuses études [11].

3.2 Adhésion et prolifération des CSMh sur des films minces de TiO_2 , Al_2O_3 et VO_x

Dans cette partie du chapitre 3, les résultats sur l'influence de divers paramètres de dépôts sur le comportement cellulaire seront présentés. Tout d'abord, les effets de la pression d'oxygène et de la température de dépôt seront discutés. Le point abordé par la suite est lié à l'influence de la composition des films minces sur la réponse cellulaire.

Dans ce sous-chapitre, il a été montré comment utiliser des films minces à base d'oxydes pour la culture et l'étude de cellules souches mésenchymateuses humaines. Les couches minces de TiO_2 , Al_2O_3 et VO_x ont été déposées avec succès par la technique de dépôt par ablation laser pulsé, et leur impact sur l'adhésion et la prolifération de CSMh a été évalué. D'après les résultats, on peut conclure que les couches minces de TiO_2 et d' Al_2O_3 permettent aux CSM d'adhérer, de croître et de proliférer dans de bons états. Pour TiO_2 et Al_2O_3 , la légère différence parmi les résultats des expériences peut être expliquée par la différence de mouillabilité, considérée comme la propriété de surface ayant une influence significative sur le comportement cellulaire. Les surfaces hydrophiles sont considérées comme préférentielles pour les cellules en général [38]. Cependant, malgré le fait que la surface de VO_x soit plus hydrophile que la surface de TiO_2 et Al_2O_3 , elle provoque apparemment une inhibition de la prolifération des CSM. Par conséquent, cette observation ne peut s'expliquer que par l'effet de la chimie de surface. Compte tenu du fait que nous avons utilisé des films minces avec une rugosité similaire de 0.1-0.3 nm, on peut s'attendre à ce que la mouillabilité des films dépende aussi principalement de sa composition chimique. En ce qui concerne l'oxyde de vanadium, l'une des explications possibles de son effet cytostatique sur les CSM est la libération de dérivé de vanadium soluble au contact du milieu de culture. Par exemple, une solubilité élevée du pentoxyde de vanadium (V_2O_5) peut avoir un effet toxique en raison de la libération d'ions vanadate [39]. Cependant, certaines études ont montré qu'à des concentrations relativement faibles de ces ions libérés (jusqu'à 10 μM), les composés du vanadium peuvent être efficaces pour améliorer la croissance cellulaire [40, 41].

En plus de l'ensemble principal d'expériences in vitro, des films minces de CuO , MgO et ZnO déposés à 100°C ont été utilisés pour estimer leur interaction avec les CSM. Comme

on peut le voir sur la Fig. 3.22a, il existe une différence considérable dans la quantité de cellules après 4 jours de culture, entre CuO et deux autres films minces. En outre, une morphologie cellulaire particulière avec des caractéristiques sphériques a été observée sur CuO, ce qui pourrait également être intéressant à explorer. Ces résultats illustrent enfin les avantages d'une grande variété de compositions de films d'oxydes, ce qui permet d'obtenir des informations complémentaires sur l'influence de la chimie de surface sur la réponse cellulaire dans le cadre d'une étude unique.

En général, l'interaction entre le matériel et les cellules souches peut être influencée par de nombreux paramètres. En ce qui concerne les propriétés des matériaux, un certain nombre de rapports ont montré que la chimie, la rugosité et la mouillabilité des surfaces, jouent tous un rôle majeur dans la réponse cellulaire. Dubey et al. ont illustré que la charge de surface est parmi d'autres facteurs qui ont un effet sur le comportement des cellules [42]. Ils ont montré comment l'application d'impulsions électriques peut également stimuler la croissance et la prolifération des cellules. Certaines études ont identifié la densité de dépôt et le temps de culture en tant que paramètres pouvant affecter la prolifération et les caractéristiques des cellules [43-45]. De plus, différents types de cellules peuvent se comporter différemment sur la même surface. Par exemple, les cellules polaires ressemblant à des fibroblastes, comparées aux cellules épithéliales non polaires, possèdent une plus grande dépendance à la chimie de surface avec une transition abrupte possible [46].

Des études ont cependant démontré que la relation entre les propriétés de surface et la réponse cellulaire n'est pas toujours simple [47]. Globalement, il semble probable que pour obtenir le meilleur résultat en termes de performance de biomatériau, une combinaison appropriée des propriétés mentionnées ci-dessus soit généralement la plus efficace. Par exemple, Vandrovcova et al. ont montré comment l'adhérence, la croissance et la maturation phénotypique de cellules MG63 étaient contrôlées par l'interaction entre la chimie du matériau et la topographie de surface [48]. En conclusion, bien que de nombreuses études récentes se concentrent sur l'utilisation de nanomatériaux ou de matériaux à surface modifiée ou traitée, notre objectif était ici de démontrer que des couches minces d'oxydes

binaires pouvaient aider à comprendre l'effet de la chimie sur la réponse cellulaire.

3.3 Différenciation des CSMh sur des films minces de TiO_2 , Al_2O_3 et VO_x

Dans cette partie du chapitre, les résultats de la différenciation des CSMh ensemencés sur les films minces d'oxydes sont décrits. La différenciation est contrôlée au moyen d'une coloration histologique et d'une réaction en chaîne par polymérase en transcription inverse en temps réel (RT-PCR). L'analyse de la différenciation cellulaire, même si elle n'en est pas au stade final, permet d'obtenir des informations quant au devenir des cellules souches, c'est-à-dire la prédisposition des cellules à devenir un certain type de cellules. Dans le cas des cellules souches mésenchymateuses dérivées de la moelle osseuse humaine, leur potentiel de différenciation en ostéocytes (cellules osseuses) et en chondrocytes (cellules constituant le cartilage) est particulièrement intéressant en termes de médecine régénérative et d'ingénierie tissulaire. Nous nous sommes donc concentrés notamment sur l'analyse de la différenciation ostéogénique et chondrogénique des CSM. De plus, leur potentiel adipogène a également été évalué.

En résumé, les résultats des tests de coloration par différenciation et de l'analyse PCR en temps réel sont en bon accord; les deux indiquant le potentiel de films minces d'oxydes pour influencer le destin des cellules souches mésenchymateuses. Les résultats concernant l'effet des films minces de TiO_2 et d' Al_2O_3 sur la différenciation ostéogénique des CSM obtenus dans le cadre de cette étude sont cohérents avec les données disponibles dans la littérature. En plus de ce qui a été rapporté précédemment, nous avons démontré que même des films minces lisses de TiO_2 et Al_2O_3 non poreux peuvent non seulement soutenir le potentiel ostéogénique des CSMh, mais également jouer un rôle dans l'induction de leur différenciation ostéoblastique. En ce qui concerne la différenciation chondrogénique qui a également été évaluée au cours de notre étude, un effet similaire du TiO_2 a déjà été rapporté [50]. Cependant, Kim et al. ont étudié le TiO_2 sous forme de nanotubes, alors que l'étude

actuelle porte sur l'utilisation d'oxydes sous forme de films minces. À notre connaissance, aucune étude n'a été réalisée sur l'impact potentiel de l' Al_2O_3 et de la VO_x , en particulier sous la forme de revêtements minces, sur la différenciation chondrogénique des CSMh.

Des études ultérieures seraient utiles pour voir comment les films minces d'oxydes affectent le chemin de différenciation cellulaire après des cultures cellulaires à long terme. Ceci, à son tour, pourrait être obtenu en modifiant la quantité de changements de milieu et en réduisant également la valeur de la densité cellulaire du placage, car dans notre cas, la durée de la culture cellulaire était limitée par le point où les cellules atteignaient la confluence. De plus, il a été démontré dans d'autres études que la densité cellulaire initiale pouvait elle-même être un facteur d'influence de l'interaction cellule-matériau, et de sa différenciation [61]. Globalement, de nombreuses méthodes et approches peuvent être utilisées pour diriger les cellules souches vers un chemin de différenciation particulier. La chimie de surface et la topographie sont les paramètres clés qui peuvent être efficaces pour contrôler la différenciation des cellules souches [62-64]. Un certain nombre d'études ont démontré qu'une variation de la rigidité du substrat peut également influencer sur le devenir des cellules souches [65, 66]. De plus, la forme d'une cellule souche peut également influencer l'engagement de sa lignée [67]. Comme indiqué au sous-chapitre 3.1, il existe une petite différence d'hydrophilie des films minces susceptibles de provoquer la propagation des cellules et éventuellement, parallèlement à la différence de composition chimique de la surface des films, de l'expression de divers gènes.

3.4 Influence des couches minces d'oxydes métalliques sur le comportement des cellules cancéreuses

Afin d'explorer plus avant la possibilité d'utiliser des couches minces d'oxydes pour étudier les interactions cellule-matériau, nous avons évalué également l'adhésion et la prolifération de plusieurs lignées de cellules cancéreuses avec des couches minces de ZnO , CuO , VO_x , Al_2O_3 et TiO_2 déposées sur des substrats de verre. Un substrat de verre non recouvert et

du plastique sont toujours utilisés comme témoins. Ce dernier, puit plastique, est un choix commun comme substrat pour les cultures cellulaires car il est spécialement prétraité pour faciliter la fixation et la croissance des cellules.

Dans nos expériences, nous avons choisi des lignées cellulaires associées à leur résistance au traitement couramment appliqué: IGROV1-R10 et SKOV3 sont résistants au cisplatine utilisé en chimiothérapie, tandis que les lignées cellulaires de chondrosarcome sont radiorésistantes [75, 76]. Les résultats que nous avons obtenus indiquent que les films minces à base d'oxydes, tels que VO_x et TiO_2 , peuvent être utilisés en culture primaire. Il serait intéressant de rechercher si ces cellules se comportent différemment sur les films minces par rapport à leur comportement dans la tumeur. L'effet positif de certains oxydes sur la prolifération cellulaire pourrait également être intéressant du point de vue de l'application à la technologie dite organoïde, qui est associée à la formation d'organoïdes à trois dimensions simulant les caractéristiques d'une tumeur. Une telle approche attire actuellement beaucoup d'attention, car elle peut fournir un système permettant de mieux comprendre la réponse aux médicaments et d'optimiser les traitements du cancer [77, 78].

4 Conclusions and perspectives

Ces travaux sont concentrés sur l'utilisation de couches minces d'oxydes métalliques (TiO_2 , Al_2O_3 et VO_x) comme substrats pour la culture de cellules humaines (cellules souches mésenchymateuses dérivées de la moelle osseuse, CSMh et cellules cancéreuses). Les films minces d'oxydes sont choisis comme substrats car ils peuvent non seulement affecter la biocompatibilité du matériau et améliorer ses propriétés mécaniques, mais peuvent également servir d'inhibiteurs de la libération d'ions potentiellement nocifs. Les très faibles valeurs de rugosité de surface (0.1-0.2 nm) des couches minces produites par la technique de dépôt par laser pulsé (PLD) ont permis d'étudier l'influence de la chimie de surface uniquement. Cette approche a permis de suivre la relation entre la composition des films minces d'oxydes et la réponse cellulaire correspondante.

En ce qui concerne l'interaction des CSMh avec les films minces de TiO_2 , Al_2O_3 et VO_x , leur adhésion, leur prolifération et leur différenciation ont été évaluées. Tout d'abord, nous avons pu montrer que les films minces d'oxyde de TiO_2 et d' Al_2O_3 n'interféraient pas avec l'adhérence et la prolifération normales. À son tour, un effet cytotatique possible des films minces VO_x pourrait être observé. Deuxièmement, au moyen d'analyses en temps réel de la réaction en chaîne de la polymérase de la transcriptase inverse (RT-PCR) et de la coloration, l'effet des films minces d'oxydes sur le chemin de différenciation des cellules souches mésenchymateuses a été démontré. Bien que d'autres expériences soient nécessaires pour explorer ces effets, les résultats ont révélé que les films minces à base d'oxydes peuvent effectivement affecter la différenciation des CSM. Les résultats indiquent notamment le potentiel d'utilisation de couches minces de TiO_2 et d' Al_2O_3 pour orienter les CSM vers des voies de différenciation ostéogéniques et chondrogéniques, qui sont pertinentes pour l'application des CSM en médecine régénérative et en génie tissulaire.

La différence d'adhésion, de prolifération et de différenciation cellulaire dans le cas des cellules souches peut être associée à l'influence de la chimie de surface et, par conséquent,

à sa mouillabilité. Ce phénomène de surface peut être responsable à la fois de la variance de l'adsorption des protéines et de la surface de propagation des cellules, qui font partie des paramètres affectant le comportement des cellules.

Une autre partie de la présente étude portait sur l'étude de l'influence des films minces d'oxydes sur le comportement des cellules cancéreuses. Deux lignées de cellules cancéreuses de l'ovaire (IGROV1-R10, SKVO3) et plusieurs oxydes de métaux (ZnO, CuO, VO_x) sous forme de couches minces ont été utilisés pour ces tests. Les résultats de ces expériences montrent l'effet positif des films minces de VO_x sur l'adhésion et la prolifération des cellules cancéreuses, ce qui est intéressant pour l'utilisation de films minces de VO_x dans des cultures de cellules primaires. Le ZnO a un effet cytotoxique dans les deux lignées cellulaires, à savoir IGROV1-R10 et SKVO3, tandis que le CuO présente une activité cytotoxique uniquement dans le cas des cellules IGROV1-R10. De plus, l'interaction des lignées cellulaires de chondrosarcome JJ012, SW1353, FS090 et CH2879 avec les couches minces de Al_2O_3 , ZnO, CuO, TiO_2 et VO_x a été évaluée. Des différences significatives dans la prolifération cellulaire ont été observées pour certaines des combinaisons. Les résultats obtenus à la fois pour les lignées cellulaires de cancer de l'ovaire et de chondrosarcome révèlent le potentiel d'utilisation de films d'oxydes d'oxydes pour des tests de dépistage et une étude du comportement des cellules cancéreuses.

En ce qui concerne les limites du présent travail, il convient de noter qu'une période de culture cellulaire plus longue permettrait d'obtenir des données plus précises sur la coloration par différenciation et l'expression génique. L'utilisation de substrats de taille plus appropriée améliorerait également la précision des résultats. En alternative aux lames de verre, le titane et ses alliages, la céramique et de nombreux autres matériaux pour implants conventionnels pourraient également constituer un choix intéressant pour la culture cellulaire. En perspective, d'autres types de cellules (par exemple, des bactéries) peuvent être utilisés pour les cultures. Il serait également intéressant d'utiliser diverses méthodes de modification de la surface, telles que la lithographie et la gravure, pour incorporer des groupes fonctionnels, modifier la morphologie et la rugosité de la surface.

En conclusion, le comportement des cellules dépend d'un grand nombre de facteurs et l'interaction cellule-matériau peut être très sensible aux variations de l'environnement et des propriétés des matériaux. Par conséquent, il est souvent difficile de comparer correctement les résultats d'études distinctes, même s'il s'agit de propriétés chimiques analogues du matériau. Ces écarts peuvent être réduits au minimum en testant autant de compositions et types cellulaires que possible dans l'étude singulière et cela peut être réalisé en utilisant l'approche similaire à celle décrite dans notre travail. Nous pensons que les résultats obtenus constituent un complément précieux au domaine de connaissances sur l'influence des couches minces d'oxydes sur le comportement cellulaire.