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Influence of nanocrystalline structure and surface properties of TiO_2 thin films on the viability of L929 cells

Małgorzata Osękowska[1], Ewa Karuga-Kuźniewska[1], Damian Wojcieszak[2], Michał Mazur[2], Agata Poniedziałek[2], Danuta Kaczmarek[2], Maria Szymonowicz[1], Zbigniew Rybak[1]

[1] *Wroclaw Medical University, Department for Experimental Surgery and Biomaterials Research, Poland*
[2] *Wroclaw University of Technology, Faculty of Microsystem Electronics and Photonics, Wroclaw, Poland*
[*] *Corresponding author: e-mail: batyckamalgorzata@gmail.com*

In this work the physicochemical and biological properties of nanocrystalline TiO_2 thin films were investigated. Thin films were prepared by magnetron sputtering method. Their properties were examined by X-ray diffraction, photoelectron spectroscopy, atomic force microscopy, optical transmission method and optical profiler. Moreover, surface wettability and scratch resistance were determined. It was found that as-deposited coatings were nanocrystalline and had TiO_2-anatase structure, built from crystallites in size of 24 nm. The surface of the films was homogenous, composed of closely packed grains and hydrophilic. Due to nanocrystalline structure thin films exhibited good scratch resistance. The results were correlated to the biological activity (*in vitro*) of thin films. Morphological changes of mouse fibroblasts (L929 cell line) after contact with the surface of TiO_2 films were evaluated with the use of a contrast-phase microscope, while their viability was tested by MTT colorimetric assay. The viability of cell line upon contact with the surface of nanocrystalline TiO_2 film was comparable to the control sample. L929 cells had homogenous cytoplasm and were forming a confluent monofilm, while lysis and inhibition of cell growth was not observed. Moreover, the viability in contact with surface of examined films was high. This confirms non-cytotoxic effect of TiO_2 film surface on mouse fibroblasts.

Keywords: TiO_2, surface properties, nanocrystalline thin film, cell morphology, viability of L929 cells.

INTRODUCTION

Titanium dioxide has been used for years as biocompatible material. High number of devices used in dental prosthetics, orthopedics and vascular surgery proves excellent physical and chemical properties of TiO_2[1, 2]. The corrosion resistance, mechanical strength at relatively low density (approx. 4 g/cm^2) and the absence of irritating action on the tissue allow on classification of titania as a one of the most common used materials in medicine[3–6]. Nowadays, application of titanium implants is connected, among others, with oxidation of their surface[7]. Such thin oxide film changes the electric potential of the surface, increases resistance to corrosion and affects biocompatibility of the implant[7, 8]. Therefore, additional TiO_2 thin film on the top of the implant will have a positive effect on its durability (also in biological aspect). According to Taubert et al.[9] biocompatible material does not cause toxic, irritating or allergic reaction in recipient. Titanium dioxide is such material in spite of literature data presenting adverse local and systemic reactions[10–15]. Side effects due to Ti-implants are explained as oversensitivity of the organism. However, allergy to other metals such as Ni and Pd, which appear in titanium alloys in trace amounts as impurities, is possible[16, 17]. The determination of cytotoxic properties during *in vitro* studies is necessary for evaluation of new materials and selection of these with potential systemic toxicity. In this work physicochemical properties and cytotoxic evaluation of the surface of nanocrystalline TiO_2 thin films was presented.

EXPERIMENTAL PART

Sample preparation

Titanium dioxide thin films were prepared by modiffied magnetron sputtering. This process relies on the application of several modifications added to conventional sputtering, e.g. introduction of the so called hot target as an effect of applying a small ring-like gap between the sputtered round target and the magnetron cooling plane. Oxygen was used as both: the working and the reactive gas during sputtering and the pressure was kept below 10^{-1} Pa. Low pressure results in a longer mean free path of the particles reaching the substrates. Additionally, during the deposition substrates were heated to ca. 500 K. All these modifications allow for nanocrystalline TiO_2-anatase structure growth during deposition[18–20].

Structural and surface characterization

Structural properties of titania films were determined based on the results of the X-Ray Diffraction (XRD) method. For the measurements, Siemens 5005 powder diffractometer with Co Kα X-ray (l = 1.78897 Å) was used. To determine the surface topography, the AFM measurements were performed with the use of the UHV VT AFM/STM Omicron atomic force microscope operating in the ultra high vacuum conditions, in the contact mode. The physicochemical properties of the surface were also examined by X-ray photoelectron spectroscopy. XPS studies were performed to determine the chemical states of the titanium and oxygen with the aid of Specs Phoibos 100 MCD-5 (5 single channel electron multiplier) hemispherical analyzer using Specs XR-50 X-ray source with Mg Kα (1253.6 eV) beam. Measurement results were analysed with the aid of CasaXPS software. All spectra were calibrated with respect to the binding energy of adventitious C1s peak at 284.8 eV. Analysis of the surface properties was completed by measurements of contact angle and critical surface tension, carried out with a computer controlled Attension Theta Lite tensiometer. Liquids used for the contact angle determination were deionized water, ethylene glycol and ethanol. Contact angle measurements were performed according to the sessile drop method[21]. The wettability of different

solid materials can be also characterized by the method proposed by Zisman[21, 22]. Using a series of liquids with different surface tensions, a graph of cosθ vs. γ is determined. Critical surface tension equals the surface tension at which the plotted line intersects 1.0 and it is often interpreted as the highest value of surface tension of a liquid, which will completely wet the solid surface.

Optical properties

Optical properties were evaluated on the basis of transmission and reflection measurements. The experimental system was based on an Ocean Optics QE 65000 spectrophotometer and a coupled deuterium-halogen light source. On the basis of these measurements the analysis of the refractive index was performed with the aid of FTG FilmStar software using Generalized Cauchy model for materials with extinction coefficient higher than 0.

Scratch resistance

Scratch resistance of the deposited thin films was investigated using the Summers Optical's Lens Coating Hardness Test Kit. For the purpose of abrasion resistance examination, steel wool test was carried out and consisted of rubbing the surface of prepared coatings with 0 grade steel wool pad using applied load of 0.5 N. Steel wool pad was pressed to the surface of the coating with selected force and was moved across the thin film for 75 cycles. The abrasion resistance test was performed according to the well acknowledged standard[23] and literature reports [e.g.[24]]. Surface was examined for scratch resistance by optical microscope and optical profiler.

Cell culture preparation for biological test

Preparation of the cell culture for studies was based on the principles contained in the standard ISO 10993-5:2009, which describes test methods to assess the *in vitro* cytotoxicity of medical devices. Cells L929 – murine fibroblasts after thawing were twice passaged using trypsin-EDTA 0.25% (SIGMA). They were cultured with an EMEM medium with L-glutamine (ATCC) and 10% Horse Serum (ATCC), under standard conditions in constant humidified chamber – in an Steri Cycle 381 incubator (Thermo Scientific) at 37°C with 5% CO_2 addition.

Indirect method (MTT test)

Indirect test was performed using extracts of tested and control materials. Four kinds of extracts were prepared. They were performed using the following ratio - sample of material with a total area of 6 cm^2/1 cm^3 of culture medium with serum. The extracts were used with the following concentrations: 100%, 50%, 25% and 12.5%.

To detect possible errors of the experimental procedure – the positive attempt was conducted (sodium laureth solution of sulphate (SLS) in concentrations: 0.1 mg/ml, 0.2 mg/ml). In contrast, the negative control performed correctly allows on observing the effect of only one test factor. High density polyethylene HDP as recommended negative control material was used. Last of these extracts was a blind sample – culture with nutritious medium[25].

L929 cells were suspended at a concentration of $1x10^5$ cells/ml ($1x10^4$ cells/well) and seeded in 96-well cluster cell culture plates. After 24, 48 and 72 h the complete culture medium was replaced with equal volumes (100 ml/well) of experimental and control extracts. Cells with appropriate extracts were incubated by 24 h, 48 h and 72 h. After this time tested and control extracts were removed from the plates, the wells were rinsed with 100μl medium. After washing, 100 ml solution of MTT was added to each well and incubated for 2 h at 37°C in 5% CO_2.

The MTT (Thiazolyl Blue Tetrazolium Bromide) assay used in this study is a sensitive, reliable and commonly used enzymatic assay to determine the cytotoxicity and biocompatibility of biomaterials dedicated for medical devices[25–27]. The principle of MTT assay is evaluation of the ability of a living cell to perform reaction of yellow tetrazolium salt transformation into a dark blue insoluble formazan. Responsibility for the transformation falls on Succinate dehydrogenase – mitochondrial enzyme active in every undamaged cell. The amount of created formazan is directly proportional to the number of cells in culture[28]. The level of L929 cells survival in the indirect contact was determined by absorbance method with the use of Epoch (Biotek) spectrophotometer at 570 nm wavelength (reference wavelength of 650 nm).

Direct method

In direct method examined samples (TiO_2 thin films on glass substrates) in size of 1 cm^2 were used. Cells were seeded on their surface at concentration of $1x10^5$ cells/ml. The incubation time was 24, 48 and 72 h. The level of cells survival was measured with automated cells counter – ADAM. It utilizes a LED based fluorescence microscope, CCD detection technologies. ADAM is based on a fluorescent dye – Propidium Iodide (PI). PI does not enter cells with intact cell membranes or active metabolism. In contrast, cells with damaged membranes or with inactive metabolism are unable to prevent PI entering the cell. As a result, the nuclei of non-viable cells are onlystained. Qualitative information in form of photographic documentation was obtained with the use of reverse-phase contrast microscope CKX 41 (Olympus). Morphological evaluation of cells was performed according to toxicity scale considering the proper criteria[25]. Continuous recording of morphological changes, cell growth and death provide reliable data. JuLI Br Live Cell Movie Analyzer allowed for monitoring the dynamics of cell proliferation of mouse fibroblasts. Pictures were taken every minute for 24 hours. All observations were carried out on the line L929 (NCTC clone 929) reference cell line from American Type Culture Collection[25]. The use of L929 – murine fibroblast – is preferred in *in vitro* toxicity assessment of biomaterials due to the easiness of their amplification or relatively consistent and well-known behavior[29, 30]. After absorbance test L929 cells survival level was calculated with relation to the control, which was taken as 100%, according to the equation[25]:

$$V = \frac{pB}{pK} \cdot 100(\%) \tag{1}$$

where: V – survival level, pB – average absorbance values for the test sample, pK – average absorbance values for the control.

RESULTS AND DISCUSSION

Structural and surface properties

The XRD patterns for as-prepared thin films are shown in Figure 1. Undoped, as-deposited titanium dioxide has nanocrystalline structure dominated by the anatase phase with an average crystallite size of approximately 24 nm. Determined crystallites sizes might be encumbered with small error, while the Signal-to-Noise ratio of registered XRD pattern was rather poor. A negligible shift of the diffraction peak related to the (101) anatase crystal plane towards lower angle (2q), as-compared to the standard Powder Diffraction File[31], indicates presence of a tensile stress.

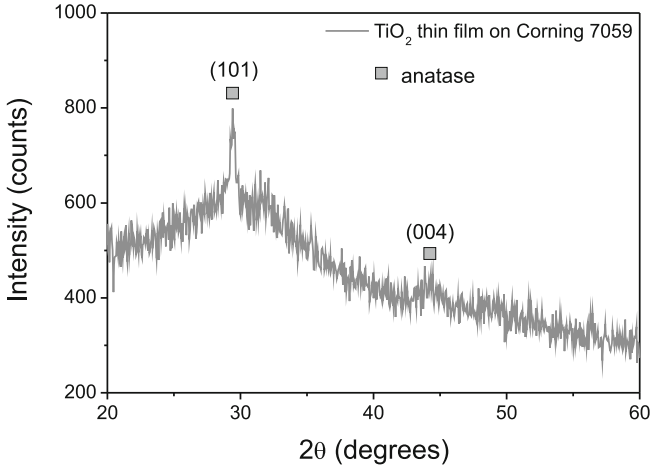

Figure 1. XRD pattern of as-deposited titania thin film on Corning 7059 glass substrate

AFM investigation was performed in order to gain information related to the surface topography of the as--prepared TiO_2 thin films. The three-dimensional AFM images with various X-Y scales are shown in Figures 2a, b. The surface of deposited thin films was crack--free, composed of visible grains and densely packed, whose maximum height was approximately 24.4 nm. The height distribution of as-prepared coatings is presented in Figure 2c. The results show a symmetric height distribution in the samples thus testifying about the good homogeneity of their surface. Moreover, the calculated root mean square (RMS) surface roughness was small and equal to ca. 2.9 nm. Additionally, the cross-section surface profile (Fig. 2d), marked in three-dimensional image in Fig. 2b, showed that visible grains were of round shape.

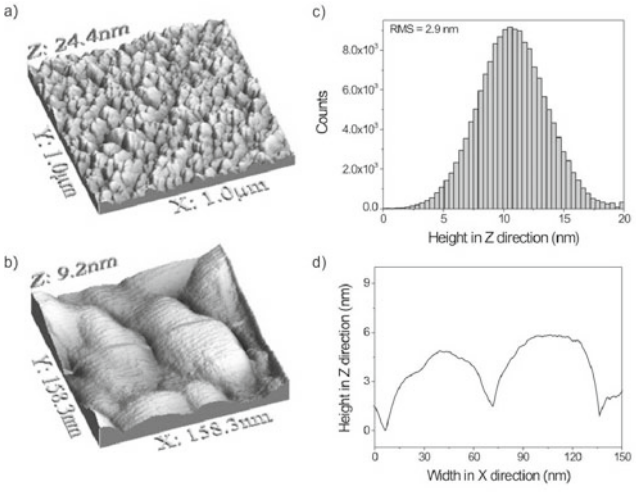

Figure 2. AFM images of TiO_2 thin films surface (a, b) with height distribution of grains size in Z direction (c) and cross-section profile of the surface (d)

XPS measurements were performed to determine the chemical states of the titanium and oxygen on the surface of deposited TiO_2 thin films. In Figure 3 a, b the Ti2p and O1s core level spectra are presented, respectively. Spectroscopic parameter which can be related to the stoichiometry of the Ti(IV) is the difference between the binding energies of $Ti2p_{3/2}$ and $Ti2p_{1/2}$ and should be equal to approximately 5.6–5.7 eV[32–34]. For deposited thin films the position of the Ti2p doublet and the separation energy width equal to 5.7 eV between the $Ti2p_{3/2}$ and

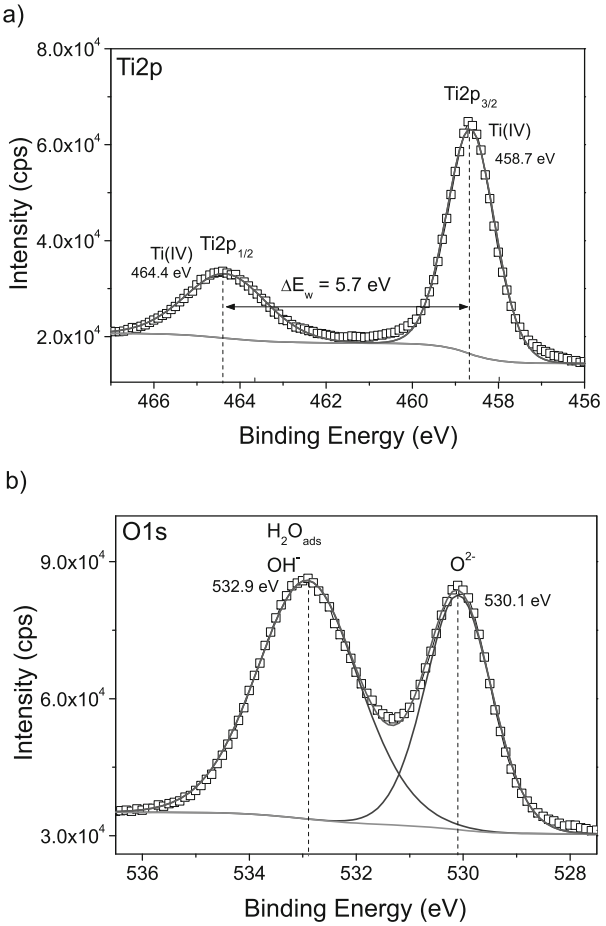

Figure 3. XPS spectra of a) Ti2p and b) O1s core levels for TiO_2 thin films

$Ti2p_{1/2}$ peaks indicate the Ti^{4+} oxidation state, which as a result testifies to the formation of TiO_2[35].

Results obtained for the O1s oxidation state revealed that on the surface of the thin films, water molecules (H_2O_{ads}) and hydroxyl radicals (OH^-) were adsorbed. The summarized level of adsorbed water molecules and hydroxyl radicals is equal to ca. 60.9%, which could indicate good wettability and water adsorption of thin films surface. Presented O1s results also confirmed the presence of Ti(IV) species on the surface of the coating.

The water contact angle was equal to 70.1°, therefore TiO_2 thin films can be considered as hydrophilic one (Fig. 4). In case of ethylene glycol and ethanol the contact angle was equal to 56.4° and 9.9°, respectively. Based

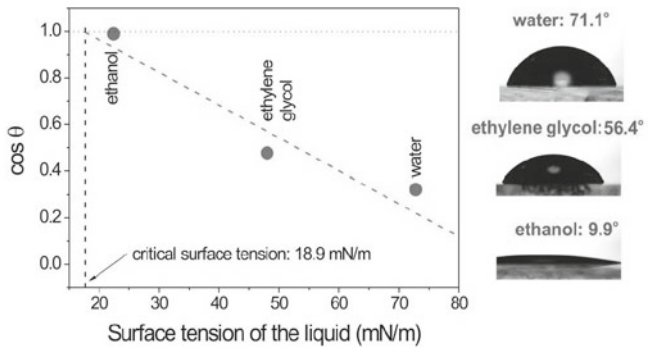

Figure 4. The results of contact angle measurements (for water, ethylene glycol and ethanol) and the graph of critical surface tension (marked with Zisman method) for as-prepared TiO_2 thin film

on these results critical surface tension was calculated and it was approximately 18.9 mN/m. Such value proves good surface wettability of nanocrystalline TiO_2 also for liquids other than deionized water. It is worth to emphasize that according to Taubert et al.[9] materials with water contact angle around 60° favour cells adhesion. That is one of the factors why the surface of prepared titanium dioxide coating should have a positive effect on a cell adhesion.

Optical properties

The analysis of optical properties has shown that prepared titania films were well transparent in visible light range. Based on transmission (T_l) and reflection (R_l) characteristics it can be noticed that average transparency level was equal to ca. 80% (Fig. 5a). The value of the cut-off wavelength ($l_{cut-off}$) was approximately 330 nm. On the basis of transmission investigations, the refractive index and thickness were determined for deposited thin films. Results for the refractive index are shown in Fig. 5b. The value of the real part of the refractive index is equal to 2.15 at the wavelength l = 550 nm. Such value is similar to reported for anatase structure in the literature [e.g. 36]. Moreover, the calculated thickness of thin films is equal to 365 nm, which is in good correlation with the results obtained by optical profiler.

Scratch resistance

In case of coatings deposited on the surface of biomaterials their scratch resistance is one of the most important properties. For this reason investigation of scratch resistance was carried out. The three-dimensional profiles (Fig. 6a) showed that the surface of TiO_2 films before the scratch test was homogenous with low roughness (1.5 nm). After the steel wool test had been performed,

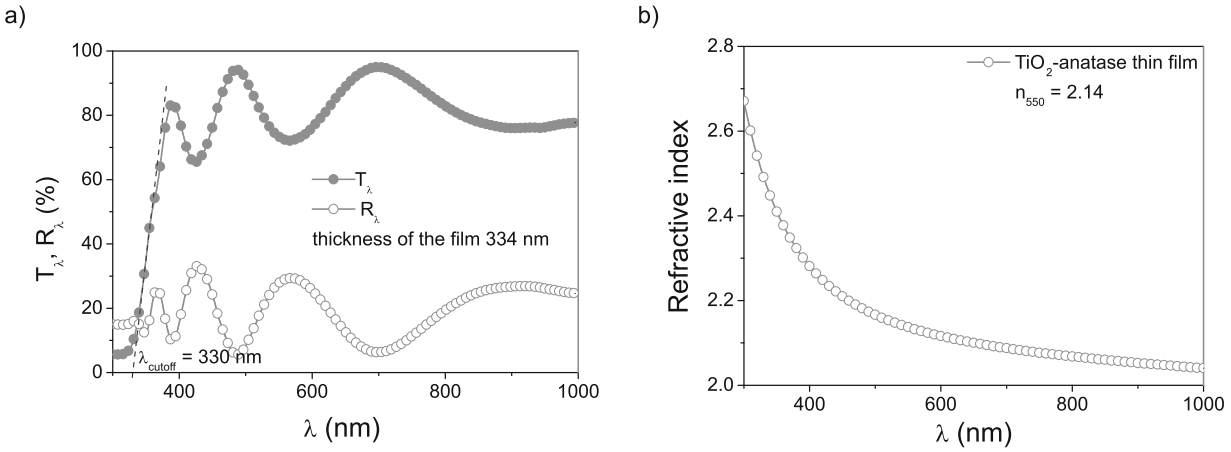

Figure 5. Characteristics of a) transmission and reflection of light and b) refractive index of TiO_2 thin film on Corning 7059 glass substrate

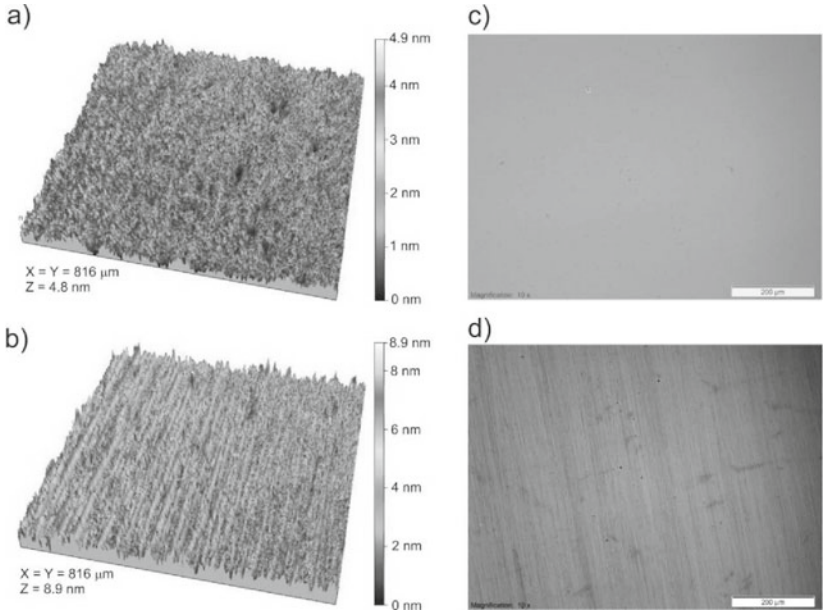

Figure 6. Results of surface geometry measurements of TiO_2 thin films before (left side) and after (right side) scratch tests

the surface roughness increased of approximately 25%, up to 1.9 nm (Fig. 6b). The evaluation of films surface was also performed with an optical microscope before and after scratch test (Fig. 6c, d). The results of microscopy observations show negligible traces of scratches, which were visible on the thin film surface (Fig. 6d). Moreover, scratches are also clearly visible in the surface profile of the investigated film, however they are very shallow, their depth does not exceed 10 nm and its amplitude remained low (Fig. 7). It proves quite good scratch resistance of the prepared titania film.

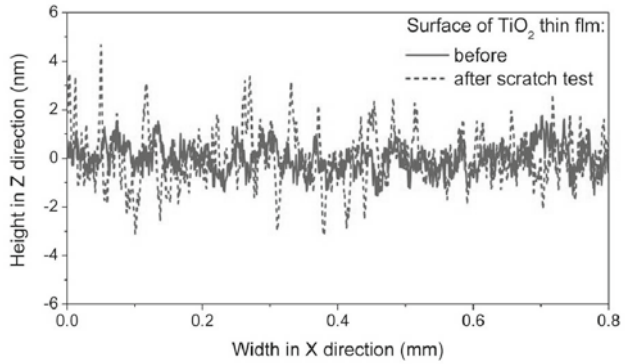

Figure 7. Two-dimensional surface profiles of TiO_2 thin film before and after scratch test

Biological (*in vitro*) studies

L929 cells were exposed to TiO_2 samples, TiO_2 extracts (100%, 50%, 25% and 12.5%) and control materials for 24, 48 and 72h and cytotoxicity was determined with the MTT assay (Fig. 8). The level of cell survival in contact with the tested material was compared with the level of cells survival incubated in a neutral environment. The amount of metabolically active cells of the control was considered as 100% survival rate. Actual number of metabolically active cells after contact with TiO_2 did not decrease below 70% compared to control[25]. After 24 hours of incubation the level of survival reached 96%. After 48 hours cell viability in relation to control was slightly decreased to 95%. The highest level of cell survival (118%) was observed after 72 h of incubation in the extracts. Lack of inhibition of cell viability below 70% indicates absence of TiO_2 cytotoxicity. During experiments lysis and inhibition of growth was not observed, only single cytoplasmic granulations and lack of inhibition of cells growth were ascertained – cells morphology was proper for L929 line (Fig. 9a, b). Extract concentration did not show influence on cells survival and morphology. Moreover, results of the direct contact showed lack of cytotoxic action of the tested material (Fig. 9c, d). Cells grown directly on TiO_2 samples characterized by proper morphology and viability compared to control materials.

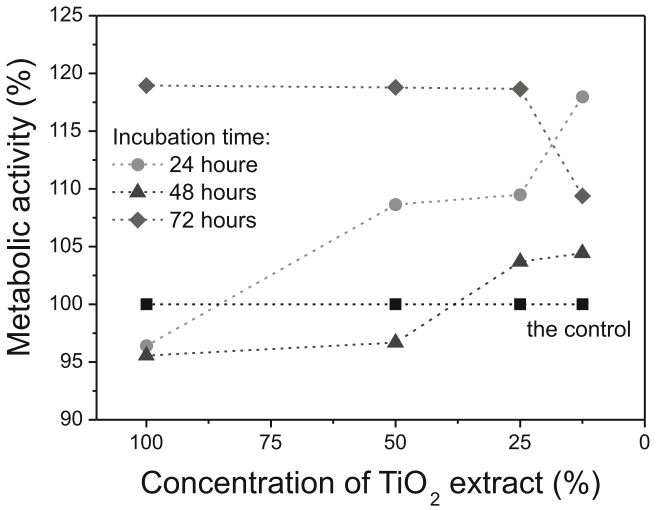

Figure 8. The level of metabolic activity of L929 cells in contact with the TiO_2 extracts after 24, 48 and 72 h of exposure

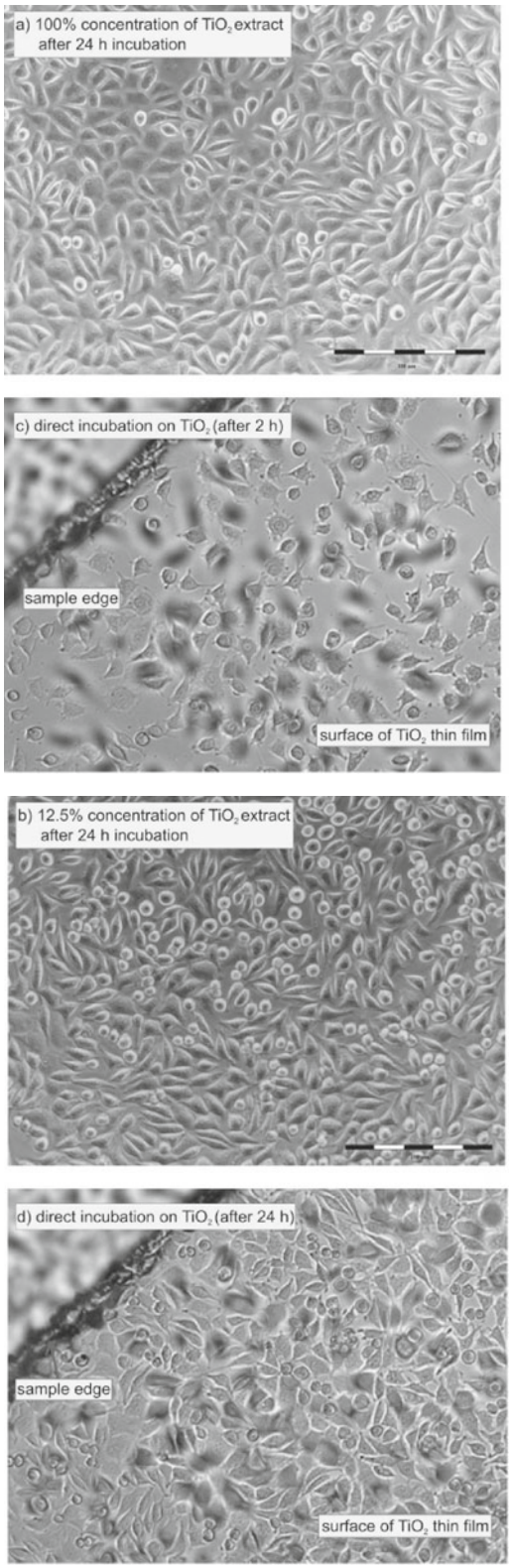

Figure 9. Cells L922 line exposed (via 24 hours) to different concentrations of TiO_2 extract: a) 100%, b) 12.5%; with cell proliferation on the film after: c) 2 and d) 24 hours of incubation

Obtained results demonstrate that TiO_2 does not show cytotoxic action in relation to murine fibroblasts L929.

Physicochemical properties of thin-film coatings such as topography, wettability and chemical composition can influence on cellular response. According to Chang et al.[37], these parameters affect the cytoskeleton organization and are crucial in the evaluation of cytotoxicity. In case of thin films many factors may influence on surface properties. It was found that roughness lower than 1 mm and hydrophilic character of the surface promotes cell adhesion[38, 39]. According to Wachesk et al.[40] increase of mitochondrial activity (MTT, LDH) and decrease of LDH activity testify about a positive cellular response of L929 to contact with the surface of TiO_2 thin films. Moreover, the results of MTT assays used for screening potential of biomaterials and predict systemic toxicity[25, 41, 42], confirmed the lack of TiO_2 cytotoxic effect also in relation to other cell lines — PC12 (adrenal glands of a rat in the medulla of chromaffinite tumour)[43] and BEAS-2B (human bronchial epithelial cells)[44]. Although, modification of the TiO_2 properties can also result in receiving of cytotoxic and antimicrobial effect, which are dependent mainly on its concentration[45], particle size[46] and crystalline phase[47]. Titanium dioxide thin films usually occur in a form of anatase or rutile. In case of the anatase phase the toxic action appears through release of LDH (lactate dehydrogenase) leading to the cell death. While, the rutile phase causes an increase of ROS production (reactive forms of oxygen) and appearance of oxidative stress, which lead to apoptosis induction[48]. However, performed investigations showed that magnetron sputtered TiO_2 thin films with nanocrystalline anatase structure did not reveal cytotoxic effect and had positive influence on the viability of L929 cells.

SUMMARY

The investigation results revealed that as-prepared TiO_2 films had nanocrystalline anatase structure with average crystallites size of 24 nm. The surface of the films was homogenous, composed of closely packed grains and hydrophilic. Due to nanocrystalline structure thin films exhibited good scratch resistance. Moreover, they were well transparent in visible light range. During *in vitro* tests lack of toxic action was shown, which makes deposited TiO_2 thin film a potential biomaterial and allows it to be qualified for further biocompatibility tests. The viability of cell line upon contact with the surface of nanocrystalline TiO_2 film was comparable to the control sample. L929 cells had homogenous cytoplasm and were forming a confluent monofilm, while lysis and inhibition of cell growth was not observed. Moreover, the viability in contact with surface of examined films was high. This confirms non-cytotoxic effect of TiO_2 film surface on mouse fibroblasts.

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