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## Cell adhesion on biomaterial surfaces with nanocomposite oxide coatings

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*Нанокмпозитні покриття на базі оксидів алюмінію, цирконію та титану мають унікальні властивості: високу індуктивність, щільність, біологічну та хімічну інертність, що дуже важливо для використання цих матеріалів як імплантатів та матриць-носіїв в клітинній інженерії. Поверхня з нанокмпозитним оксидним покриттям демонструє високу електричну інертність та діелектричні константи. Біологічна апробація новітніх нанокмпозитних матеріалів та покриттів як перспективних біоматеріалів дозволяє запропонувати принципово нові методи лікування багатьох складних захворювань.*

*Нанокмпозитные покрытия на основе оксидов алюминия, циркония и титана обладают уникальными свойствами: высокой индуктивностью, плотностью, биологической и химической инертностью, которые очень важны при дальнейшем их использовании в качестве имплантатов и матриц-носителей в тканевой инженерии. Поверхность с нанокмпозитным оксидным покрытием демонстрирует высокую электрическую инертность и диэлектрические константы. Биологическая апробация современных нанокмпозитных материалов и покрытий как перспективных биоматериалов позволяет предлагать принципиально новые методы лечения многих сложных заболеваний.*

**Key words:** cell adhesion, biomaterial surfaces, nanocomposite oxide coatings

Deposition of functional coatings is one of the most optimal ways of surface modification enabling an efficient control of stoichiometry, impurity elements, functional groups and surface charges. Nanocomposite coatings based on Al, Zr, Ti oxides exhibit unique properties: high inductivity, density, bio- and chemical inertness [1–3], which are very important for subsequent implant and tissue engineering applications. Corrosion is one of the major processes that cause problems when metals and alloys are used as implants in the body [4]. Corrosion of implants in the aqueous medium of body fluids takes place via electrochemical reactions [5], and it is necessary to understand the electrochemical principles that are most relevant to the corrosion processes. The body fluid environment may well decrease the fatigue strength of the metal implant and enhance the release of iron, chromium, nickel,

titanium ions, these ions being found to be powerful allergens and carcinogens [6]. Presence of titanium in the surrounding tissues of these implants in the form of titanium compounds and subsequent failure of implants due to fatigue, stress corrosion cracking and poor wear resistance have been reported [7, 8]. Release of metal ions into the tissues adjacent to the implants results in accumulation of harmful products in tissues and internal organs of animals [9]. Effects of substrate material composition, electrochemical properties, surface chemistry and topography on cell adhesion and proliferation processes have been largely studied [10–12]. In vitro studies of cell response to artificial material surface are basic tools to recognize the cell/material interaction regularities. The distance between phospholipid bilayers of cell membranes is of the order of 20–40 nm [13]. As the a few hundreds nanometers

of cellular receptors interaction is a few 100 nm, the surface relief modifications should be also controlled at a nanometer-submicronic level. Therefore an important task of modern methods of coating deposition consists in an effective control over chemical composition and processes of surface topography formation on the nanostructural level.

## Material and methods

Substrates for deposited coatings were stainless steel (AISI 321) and titanium-based material (Ti4Al6V) samples. The substrates were cleaned in an ultrasonic bath with standard technology. Nanocomposite  $\text{Al}_2\text{O}_3$  magnetron sputtering (MS) deposition was performed in a high vacuum pumping system with a base pressure of about  $10^{-3}$  Pa. The main details of the magnetron and ion source in the sputtering chamber were demonstrated. The structure and composition of the coatings, adhesion properties, hardness and elastic modulus, thickness were evaluated by standard methods [14].

Surface roughness was estimated by Hommel T-2000 profilometer measurements. Surface free energy (SFE), its polar and dispersion components were determined by means of Wu and Owens-Wendt-Rabel-Kaelble methods.

Corrosion examinations of anodic polarization by potentiodynamic method, Tafel and Stern curves, and impedance method in simulated body fluid (SBF) solutions were made by Potentiostat PARSTAT 2263 (AMETEK, USA). Over a frequency bandwidth of interest the impedance was presented in different ways by both the Nyquist and Bode plots. The surface topography was investigated by SEM (HITACHI, Japan) and AFM (Quesant Instrument Corporation, USA).

Cytotoxicity and cytocompatibility were estimated at in vitro tests. Cytology, morphology and proliferation activity were determined in the process of cell cultivation (fibroblasts) with coated and uncoated samples after 24 h and 3, 5 days of cultivation. The analysis of cell adhesion on substrates was made by means of SEM and AFM methods.

## Results and discussion

Corrosion parameters for nanocomposite coating  $\text{Al}_2\text{O}_3$  deposited on stainless steel (AISI 321) and titanium-based material (Ti4Al6V) samples were analysed. Corrosion examinations of anodic polarization by the potentiodynamic method at the potential range of  $-1.0\text{ V} \div +2.0\text{ V}$  with the scanning rate of  $1\text{ mV/s}$ , Tafel  $-0.050\text{ V} \div +0.050\text{ V}$  and Stern  $-0.020\text{ V} \div +0.020\text{ V}$  range curves and also by the impedance method for frequency range  $100\text{ kHz} \div 10\text{ MHz}$  in a SBF solution ( $\text{NaCl}-8.035$ ,  $\text{NaHCO}_3-0.355$ ,  $\text{KCl}-0.225$ ,  $\text{K}_2\text{HPO}_4$

$3\text{H}_2\text{O}-0.231$ ,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}-0.311$ ,  $\text{CaCl}_2-0.292$ ,  $\text{Na}_2\text{SO}_4-0.072$  at  $\text{pH} = 7.4$  and a temperature of  $37^\circ\text{ C}$ ) were made by Potentiostat PARSTAT 2263 (AMETEK, USA). The samples were immersed in an electrolyte and their potential was monitored as a function of time until the potential reached a stable value. Corrosion in the form of anodic dissolution occurs in solution like a uniform removal process for substrates and a corrosive electrolyte pore penetration process for ceramic coatings.

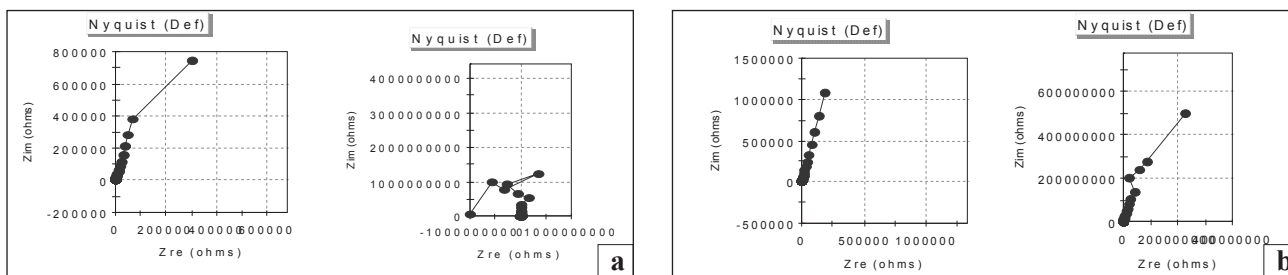
Electrochemical Impedance Spectroscopy (EIS) is a powerful analytic technique, which can provide a lot of information on corrosion reactions, mass transport and electrical charge transfer characteristics of coated materials in various solutions. Impedance spectrum describes dielectric behaviour, oxidation-reduction reactions and mass migration which are determined by electrical and chemical properties of the corrosion medium and the electrode materials. EIS spectra describe electrical charge transfer kinetics and details of physical and electrochemical corrosion characteristics of the substrate/coating interface. Platinum wire and Ag/AgCl were used as counter and reference electrodes, respectively between frequency ranges of  $100\text{ kHz} \div 10\text{ MHz}$  at a constant  $5\text{ mV}$  amplitude and  $250\text{ mV}$  initial potential for all measurements. The impedance parameters  $|Z|$ , polarization resistance  $R_p$  and capacitance  $C$  were calculated from Nyquist and Bode plots.

Impedance spectra of all the samples were recorded before and after polarization conditions, in order to evaluate the performance of the coatings under equilibrium conditions and after the onset of the corrosion process respectively. The Nyquist plot of impedance was obtained from real ( $Z_{re} = R_s + R_p / (1 + \omega^2 R_p^2 C_d^2)$ ) and imaginary ( $Z_{im} = \omega R_p^2 C_d / (1 + \omega^2 R_p^2 C_d^2)$ ) impedance at different frequencies to determine charge-transfer kinetics ( $R_s$  is electrolyte solution resistance,  $R_p$  is polarization resistance, and  $C_d$  is capacitance at interface). Fig. 1 show Nyquist plots for stainless steel (SS), SS/ $\text{Al}_2\text{O}_3$ , Ti4Al6V and Ti4Al6V/ $\text{Al}_2\text{O}_3$  coatings in the SBF solution.

The data show that coating deposition improved the charge-transfer kinetic performance on counter electrode-electrolyte interfaces.

The contact angles were measured by means of the tensiometric method. Prior to their contact angle measurements, samples were ultrasonically cleaned in acetone and deionized water, and dried. Advancing contact angles were measured by Wilhelm's method (Kruss K12) at a temperature of  $20^\circ\text{ C}$  (table 1).

Such standard liquids with well-known values of surface tension, component of dispersion and polar interaction as water, formamide, diiodomethane, ethylene glycol and  $\alpha$ -bromonaphthalene were used. Also, SFE, its polar and dispersion components were determined



**Fig. 1.** Nyquist plots for a) SS, SS/Al<sub>2</sub>O<sub>3</sub> and b) Ti4Al6V, Ti4Al6V/Al<sub>2</sub>O<sub>3</sub> in the SBF solution

**Table 1.** Average values of the advancing contact angle at a temperature of 20° C

Substrate/coating	Water, (°)	Formamide, (°)	Ethylene glycole, (°)	Diiodomethane, (°)	α-bromonaphthalen, (°)
SS/Al <sub>2</sub> O <sub>3</sub>	54.71	48.15	42.29	44.02	26.97
Ti6Al4V/Al <sub>2</sub> O <sub>3</sub>	55.67	44.00	44.77	46.17	28.04
SS	72.4	45.6	51.8	37.3	18.3
Ti6Al4V	56.5	33.2	34.5	35.3	20.8

by means of Wu [15] and Owens-Wendt-Rabel-Kaelble [16] methods (table 2).

Cytotoxicity and cytocompatibility were experimentally studied in vitro: in a culture of fibroblasts. In the process of cell cultivation with coated samples their cytology, cell morphology and proliferation activity were determined after 24 h and 3, 5 days of cultivation. Rat hypodermic tissue was extracted for obtaining initial fibroblast cultures. The suspension of extracted cells was centrifuged at 750 r/min during 15 min. The cell inoculation area was  $3 \times 10^5$  cell/ml density of culture medium. The cultivation of fibroblasts in 3 ml of Dulbecco Modified Eagle's Medium (DMEM, Sigma) supplemented with 10% foetal calf serum, 80 mg/ml penicillin, 100 mg/ml streptomycin was made by methods of monolayer culture at thermostat conditions (at a temperature of 37°C in 5% CO<sub>2</sub> atmosphere during 5 days). The cells, which adhered on the samples, were cleaned by a buffer solution (pH = 7.2) and double distilled water and fixed in 2.5% glutaraldehyde on 0.1 M buffer solution during 2h and 1% OsO<sub>4</sub> solution during 1 h. Then the samples were dehydrated in a grade series of alcohol. Cell morphology on the different coated samples was examined by SEM and AFM observations. Other samples with the adhered cells were trypsinized with 0.01% trypsin/0.5 mM EDTA. The experiments were independently triplicate.

Fig. 2 shows the adhered cell morphology on stainless steel (SS) and SS/Al<sub>2</sub>O<sub>3</sub> coatings after 3 (a, c) and 5 (b, d) days cultivation by AFM. The data present the cell adhesion behaviour. After 3 days in the culture, fibroblast were well spread both on the control and coated surfaces.

The cell morphology was typical for cells on the coated surface. After 5 days of cultivation the density of cell increased in all the samples. In the centre of any

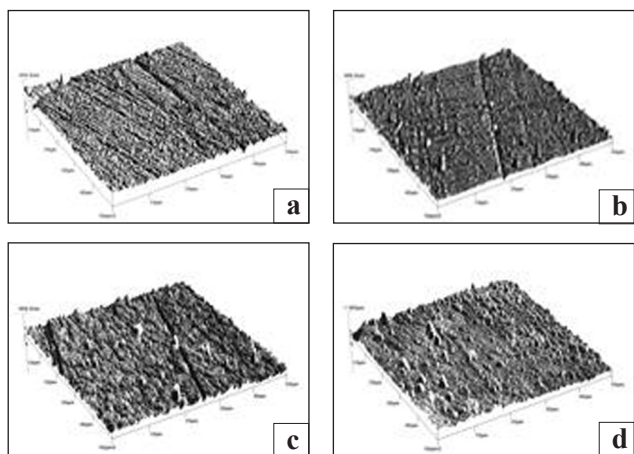
sample the density was maximal. Differences in cell attachment and spreading on different surfaces were detected. The first column for each sample shows the 1<sup>st</sup> day of experiment, and the second column shows the 5<sup>th</sup> day of experiment (fig. 3).

Previous studies have examined the effect of surface energy on cell functions, such as adhesion, proliferation and differentiation. In some cases the cell functions were enhanced on hydrophilic surfaces, in other cases on hydrophobic ones. The average cell area after 24h had its maximum values at a water contact angle of 60° [17]. In our study, the values of the water contact angle were measured in the range of 50–60° and evaluated value of SFE was within 40–50 mN/m. There is an intermediate region between hydrophobic and hydrophilic surfaces. A possible explanation of the similar cell behaviour on different substrates and oxide coating surfaces with close roughness parameters (at a range of 20–40 nm) may be in the fact of intermediate values of SFE for such coating types. A more detailed study of the effect of surface free energy on cell spreading and proliferation requires taking into account disperse and polar components of surface free energy values and fractional polarity [18].

The best adhesive parameters were obtained in the case of substrates with the highest values of the polar part component of SFE, such as Ti4Al6V and Al<sub>2</sub>O<sub>3</sub> oxide coatings (MS) (table 2).

**Table 2.** Values of total surface free energy, dispersion and polar components, fractional polarity (by Wu method for the formamide-water system at a temperature of 20°C)

Substrate/coating	σ [mN/m]	σ <sup>d</sup> [mN/m]	σ <sup>p</sup> [mN/m]
SS / Al <sub>2</sub> O <sub>3</sub>	48.61	18.89	29.72
Ti6Al4V/Al <sub>2</sub> O <sub>3</sub>	49.11	21.29	27.82
SS	43.25	26.95	16.30
Ti6Al4V	51.92	27.03	24.89



**Fig. 2.** Adhered cell morphology on SS (a, b) and SS/Al<sub>2</sub>O<sub>3</sub> (c, d) coatings after 3 (a, c) and 5 (b, d) days cultivation by AFM

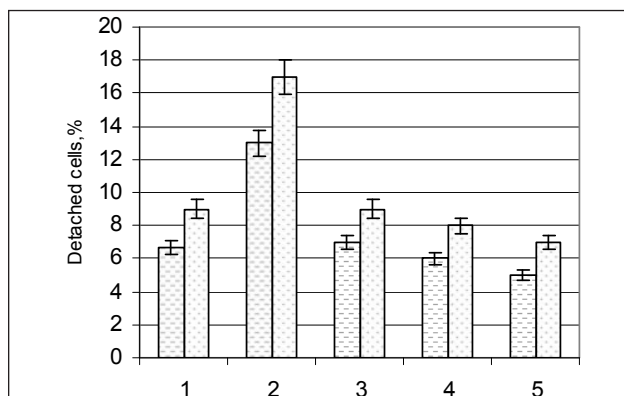
## Conclusions

Our results demonstrate that the best biological response parameters (total cell number, proliferation function, cell morphology) were obtained in the case of oxide coatings on substrate materials (SS, Ti6Al4V) with roughness parameters within 20–40 nm, intermediate values of SFE in the range of 40–50 mN/m and the highest values of the polar part component of SFE such as Ti4Al6V (24.9 mN/m) and Al<sub>2</sub>O<sub>3</sub> oxide coatings (MS) (29.7 mN/m). The corrosion test results also show that oxide coating deposition improved charge-transfer kinetic performance on counter electrode-electrolyte interfaces. The surface with nanocomposite oxide coating has a strong capacitive response due to their electrically inert properties and high dielectric constants.

The understanding of regularities and mechanisms of nanomaterial interactions with biological objects creates prospects for a direct control of such parameters as adhesion, proliferation and differentiation of cells during their culturing. The *in vitro* approbation of nanocomposite materials and coatings as promising biomaterials makes it possible to suggest principally new strategies for treating many severe diseases and developing modern nanobiomedical methods.

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**Fig. 3.** The cell number ( $P < 0.05$ ) on the surface of SS (AISI 321) and Ti4Al6V substrates with Al<sub>2</sub>O<sub>3</sub> (MS) coatings after 3 and 5 days of cultivation. 1 — control glass, 2 — SS, 3 — SS/Al<sub>2</sub>O<sub>3</sub>, 4 — Ti4Al6V, 5 — Ti4Al6V/Al<sub>2</sub>O<sub>3</sub>

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