PRELIMINARY EVALUATION OF SELECTED BIOLOGIC PROPERTIES OF TiO_2 AND SiO_2 LAYERS ON METALLIC SUBSTRATES

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Abstract

Despite of applying modern biomaterials during constructing long term orthopaedic implants, in clinical practice there are still present wide range of complications, particularly concerning matter of implant - tissue interactions. Since interaction between implant and living tissue depends mainly on biomaterial surface features, we decided to modify orthopaedic alloys to improve their biological properties.

The object of this experiment was in vitro evaluation of selected biological properties, particularly cytotoxicity of titanium alloy and 316L stainless steel substrates coated with SiO_2 or TiO_2 thin films. The coatings were synthesized by sol-gel method. Each samples was placed into mouse fibroblast culture. The cultures in presence of tested materials were maintained for three days.

We found no distinct toxic effect of tested biomaterials. We noticed increase of fibroblast proliferation in cultures with uncoated titanium and particularly SiO_2 coated titanium plates.

***Keywords**: sol-gel coating, fibroblast, cytotoxicity, orthopaedic implant*

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Introduction

Formerly approach to treatment, especially surgical treatment was limited to removing damaged or pathologically changed tissues, however nowadays the strategies are distinct. The goal is to reconstruct tissues or organs and what is more important to restore their function. To achieve these aims, there are procedures like tissue transplantations, material implantations and most promising for the future - tissue regeneration [1].

Medical procedures in trauma or diseases of loco motor system quite often base on biomaterials applications to reconstruct or time fixate damaged tissues. Despite enormous progress in biomaterial science, still their properties are not satisfying enough. Although modern prosthesis characterize very advanced construction and material structure there are still problems with limited time of their function in human body. The problem is essential in context of more and more prolonged human life and aged societies.

Implant-tissue interface interactions mostly depends on properties of the surface of biomaterial. Crucial is only several external atomic layers of implantable material [2]. One of the ideas to eliminate undesirable interactions are surface modifications; material of excellent mechanical properties, considering as relatively bioinert, coated by bioactive layer. Construction of such a composite enable to combine advantages and to eliminate disadvantages of these two different biomaterials. Thus, researchers focused on searching for new solutions to manage increasing requirements of implants, putting together highly biocompatible and bioactive biomaterial (hydroxyapatite ceramics, bioglass) with biomaterial of favourable mechanical properties, believing that it might eliminate undesirable mechanical properties of ceramics; such as fragility and low flexibility (high Young modulus), and at the same time improved biological metal properties like biocompatibility, biological activity and resistance to corrosion [1].

It is known that titanium undergoes self oxidation in air. On metal surface creates external layer of amorphous titanium oxides. This passive layer spreads more or less 5 nm deep inside metal structure consisting of TiO, Ti_2O_3, but above all rich in TiO_2 [3]. The discovery that the oxide layer is mainly responsible for good osteintegration of titanium implants4 increased interest of TiO_2. At the beginnings of seventies of the last century L. Hench [5,6], revealed results of his experiments on bioglass. Since then silicon based or consisting materials draw researchers and clinicians attention, particularly in context of bone implant applications.

In this paper we modified surfaces of the metallic materials due to improve their biological properties. We concentrated on coating of metallic materials with titanium dioxide (TiO_2) and silica (SiO_2). The oxides were synthesised by the sol-gel method. In available literature concerning this matter many promising facts and information was described, particularly in refer to reaction of materials with bone tissue [7,8,27,30]. In specified environment on modified surface of discussed materials might form hydroxyapatite layer. It was observed that In vivo strong chemical bondings created between oxide implant surface and apatite formations. What is more important these processes are definitely more pronounced when dioxides are made by sol-gel method than other methods [9]. It is so because of low temperature thermal treatment (500^{0}C) in sol-gel method, numerous hydroxyl functional groups (Si-OH, Ti-OH) retain and they are responsible for initiation apposition of calcium phosphate formations [10,11]. Titanium and silicon dioxide rough surfaces are hydrophilic, with negative surface charge, that are the features influencing on intense protein adsorption (fibronectin, vitronectin) on the implant surface. Adsorbed proteins enable connection to cellular membrane integrins – a crucial stage in process cellular adhesion [12-14]. Higher protein adsorption increases cellular adhesion. High surface energy of TiO_2 or SiO_2 coatings is an important feature positively influencing their biological properties and in these materials responsible for this phenomenon are numerous high energy OH groups on their surface. After implanting biomaterial, connective tissue capsule surrounds it. Around implants with low surface energy the capsule is relatively thick - cells instead to implant adhere to themselves, therefore, higher surface energy results thinner the capsule [15].

The object of presented research was in vitro preliminary evaluation of biological properties, particularly cytotoxicity tests of metallic plates with modified surface. These plates were made of titanium alloy or austenitic stainless steel. Their surfaces structure were modified physically (rough surface) and chemically (coated with sol-gel silica or titanium dioxide). We assessed toxic influence of metallic samples on fibroblast culture, mainly vitality and cell adhesion to specimen.

Materials and methods

In the experiment we used metal round shaped plates of 9 mm in diameter and 1 mm thick. These plates acting as basis for coatings were made of two different metal alloys. First, austenitic stainless steel plate - samples, Fe/Cr18/Ni10/Mo3 (alloy 316L), commonly applied and known for very long time in clinical practice and second, titanium alloy plates - Ti4Al6V, the most frequently used implantation materials in various medical disciplines and well known as biocompatible material with excellent contact with bone tissue, and above all distinguishing by splendid mechanical properties. Sample surface was rough, we used two different roughness; Ra (average roughness - arithmetic mean distance between peaks and valleys of the surface from its ideal form) R_a=0,63 µm and R_a=1,25 µm. Each type of metal plate was dip-coated by sol-gel silica and titanium dioxide.

The sol-gel method is a process of modifying material surface by creating oxide coatings. It's based on hydrolysis of liquid precursors, condensation and aggregation [29] (transformation of colloidal sols into solid gel). In our experiment we used sol-gel method to synthesize the sols and dip-coating method to lay on the substrates [28]. After coating of the metallic substrates, thermal treatment (up to 500^{0}C) was conducted.

For biological evaluation, test were conducted on cell cultures of mouse fibroblast (lines 3T3/Balb received from tissue bank of Institute of Immunology and Experimental Therapy Polish Academy of Sciences in Wrocław). All procedures were conducted according to ISO Pr PN – ISO 10993-5 guidelines. Cells after defrosting (frozen in 10% DMSO, in temperature - 176^{0}C) were twice passed. We used fluid culture medium consisting of - Minimum Essential Medium + Hepes, 10% FCS - Fetal Calf Serum (CAMBREX, Belgium), prepared solution produced by SIGMA (Germany) composed of L-glutamin 1 mM/ml, penicillin 100 U/ml i Streptomycin 100 µg/ml. Each specimen was culturing in incubator (KENDRO) in following conditions; 5% CO_2, temperature 37^{0}C, continuous humidifying.

To evaluate cytotoxicity we used direct tests, we put metal samples - titanium alloy and austenitic stainless steel plates coated with silica and titanium dioxide (both obtained by sol-gel method) or pure plates without coatings - into mouse fibroblast (3T3/Balb) culture. Each of 12 chamber in testing plate (FALCON) were seeded by 0,5x10^6 fibroblasts/ml. After 24 h culture, cells adhered to the chambers' ground, we removed the old mediums placing fresh one. Then metal plates were inserted into chambers. For each of three experimental groups we created control groups; fibroblast only with culture medium, without any biomaterial. Each culture were conducted 72 h, observations in optic microscope were made every 24 h, specimens were strictly described. We assessed cell number, their morphology, proliferation, number of necrotic cells. Cell number was determining in Bürkner chamber after trypan blue staining.

In first experimental group we were testing plates without coatings. Their surface were rough made by sand blasting, we obtained two grades of roughness. In second and third group we were testing metal samples coated by dioxide layers, two different surface roughness titanium alloy plates (group II) and austenitic steel - 316L (group III) covered with TiO_2 or SiO_2 coating (TABLE 1).

TABLE 1. Number of tested plates.

	steel 316L (Fe/Cr18/Ni10/Mo3)			titanium alloy Ti4Al6V		
Roughness	uncoated	SiO_2	TiO_2	uncoated	SiO_2	TiO_2
R_a 0,63	3	3	3	3	3	3
R_a 1,25	3	3	3	3	3	3

Results

In first experimental group we evaluated influence on fibroblast culture metal biomaterials without oxide coatings, but rough surface – surface modified physically. Satisfactory results were obtained in cultures with titanium alloy plates. Both titanium plates favourably affected cells (PHOTO 1c,d), number of fibroblast was remarkably higher after 48 and 72 h than in control groups. On the contrary, fibroblast cultures in contact with steel 316L (FIG. 1, PHOTO 1a and b) were less numerous than in control and titanium groups. After 48 h, in cultures with austenitic steel, particularly in cultures with plate of surface average roughness 1.25, we observed some cells detaching from chambers' ground.

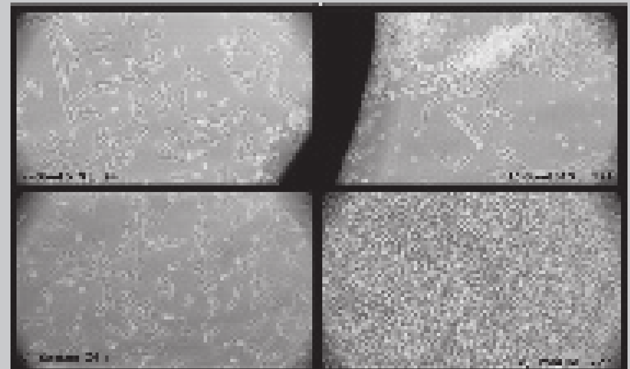

PHOTO 1. Microscopic images of fibroblast cultures with austenitic steel and titanium alloy uncoated plates after 24 and 72h.

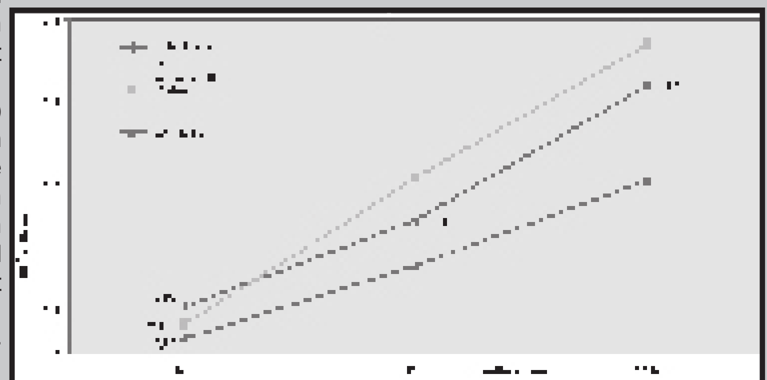

FIG.1. Comparison of I group results. Average numbers of fibroblastic cells on mm³ in cultures with austenitic steel and titanium alloy uncoated plates in relation of time and specimen.

After 72 h the process of detaching significantly increased; we observed numerous cells floating, specially close to the biomaterial specimen and fibroblast necrosis. In one case (steel 316L, R_a 1.25) cell necrosis reached 45% of total cell number and average of all cultures for this specimen was 23.25% (FIG. 2).

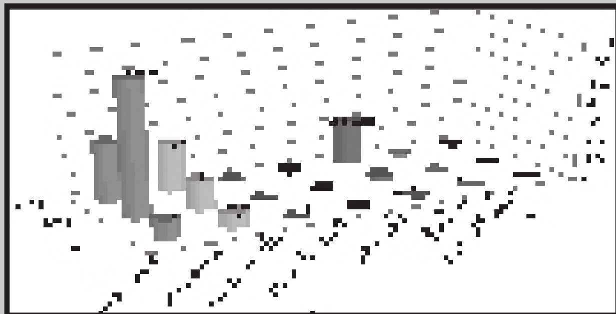

FIG. 2. Average percentage of necrotic cells during incubation with biomaterials and in control groups.

Second experimental group consist of fibroblast cultures and titanium plate with coated oxide layers SiO_2 and TiO_2. Cultures with these biomaterial specimens reached very high numbers of cells (PHOTO 2 a,b), and these values were quite similar to each other and not very distant to control group (FIG. 3). We also noticed low percentage of cell necrosis after 72 h of culture.

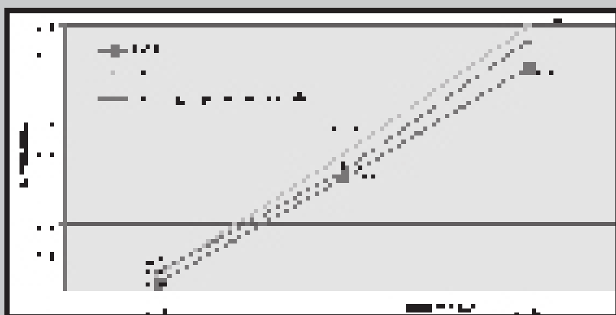

FIG. 3. Results of II group, Average numbers of fibroblastic cells on mm² in cultures with titanium alloy plates with surface coated TiO_2 and SiO_2 in relation of time and specimen.

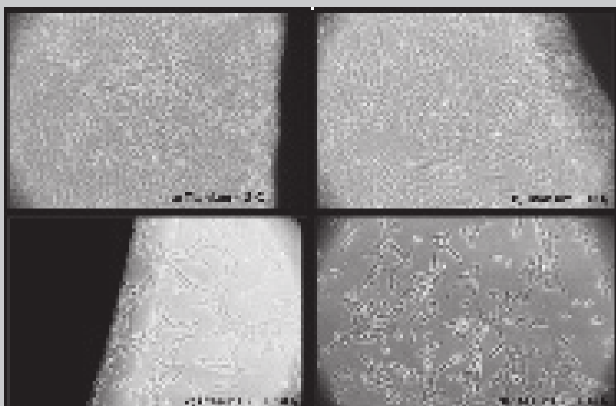

PHOTO 2. Microscopic images of fibroblast cultures with austenitic steel and titanium alloy plates coated TiO_2 or SiO_2 after 72 h. Solid dark areas on the photos are metal plates, these photos allow to observe reaction of fibroblast on direct contact with biomaterials.

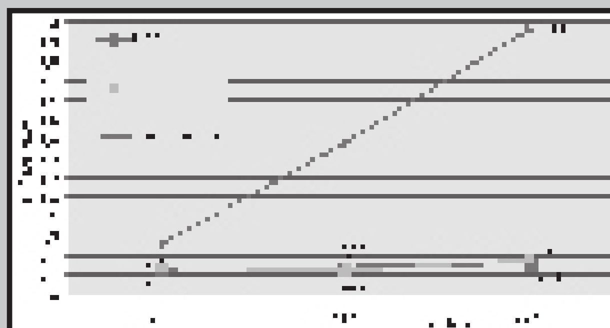

FIG. 4. Results of III group. Average numbers of fibroblastic cells on mm² in cultures with austenitic steel plates with surface coated TiO_2 and SiO_2 in relation of time and specimen.

The averages of cell deaths presented on diagram (FIG. 2) are not higher than 1.75% of cell populations in all cultures of this group. Favourably conditions were when titanium with TiO_2 samples were placed in cultures; after 72 h cell number in the culture with this specimen of average roughness 0.63 reached $2.48x10^6/mm^3$ (in FIG. 2 there are only averages of specimen groups). It was the highest result in our experiment.

In the third group we examined influence of austenitic stainless steel plates with oxide layers coatings on fibroblast cultures. All metal specimens in this group inadequately affect cell cultures, final fibroblast numbers were definitely lower than in control and other groups after 48 and 72 h (FIG. 4).

What's more important we observed that undesirable effect on fibroblasts was even expressed stronger in steel specimens with oxide layers coatings, than pure (uncoated) austenitic steel. We observed after 24 h steel plates with coatings inhibited cell proliferation, and also no fibroblast growth was noticed next days (FIG. 4). In each culture cell agglutinated, detaching from culture chamber base, shrinking and not adhering to examining metal plates (PHOTO 2). Cell necrosis reached 67,5% of fibroblast populations (FIG. 2).

In one of cultures where austenitic steel coated with silicon dioxide (R_a 1.25) was tested after 48 h revealed massive cell necrosis, without any noticeable reason. This particular case reached 100% of cell deaths and influenced average for the whole group. We suspected that contamination of surface layer caused the incident. It seemed to be confirmed by the fact that we used 6 chemically identical specimens and necrosis occurred only in one culture.

Discussion

Materials which potentially might be use for implanting purposes in medicine, before clinical trials begun it obligatory to conduct wide range of pre-clinical trials to establish some basic features like biocompatibility and biofunctionality. Crucial in preclinical stage of research on biomaterials are animal tests; good result of material evaluation in vivo, in destined tissue let researcher begin clinical trials. Current regulations require in vitro test only to establish material cytotoxicity, however present achievements of in vitro cell culturing techniques gives wide range of tests allowing obtain much more information concerning future implant properties and furthermore decrease amount of necessary animal tests [16,17].

In this stage of our research we decided to determine whether examining biomaterials are toxic. We chose direct method toxicity testing - mouse fibroblast cells culture,

because of their high sensivity on toxic factors in culture medium. Balb mouse fibroblast cell lines are useful tool in cytotoxity test because of definite and stabile cell phenotype, not changing during successive passing on the contrary to fresh isolated form tissue cells. We were comparing number of cells in control groups (cultures only with medium) with number of cells in cultures with metal plates. Differences in cell count between particular groups let us draw conclusions about grade of toxic influence of testing specimens on fibroblasts. We observed that number of cells in cultures with austenitic steel plates were lower than in control groups. These plates however did not bring to cell necrosis or strong inhibition of fibroblast proliferation in cultures with uncoated steel plates, in each culture cells outnumbered initial cell counts (number). Not only dioxide coatings did not improve culture result but even (also) worsen their final results. Stainless steel (316L) is very commonly used in orthopaedic surgery because of low material cost and relatively good biocompatibility. Though implants made of this material are usually time limited implants (for instance osteosynthesis), that is because the material in human tissues corrodes, release iron, copper or chromium compounds to local tissues and induce fibrous tissue capsule around implant18. Vallet-Reg in his experiments demonstrated that coatings created by sol – gel method on austenitic steel 316L do not inhibit enough corrosion processes as a result of the reactivity between the Cr of the steel and the Si and C of the chemical medium. Chromium from biomaterial reacts with surface located silicon and carbon from culture medium creating complex chemical compounds, leading to form surface corrosion pits19. We assume that this process is responsible for poorer results in groups testing stainless steel 316L coated by silicon dioxide.

All titanium plates; uncoated and coated influenced very favorably on fibroblast cultures. We concluded no toxic effect on fibroblasts and even proliferation stimulation in titanium SiO2 coated. Dieudonne and co-workers compared number of rat marrow cells in 5 days culture with uncoated and TiO_2 coated titanium alloy. Numbers of cells in their experiments after 3 and 5 days were the highest in uncoated titanium group, but only little less in dioxide layer coated. Authors used fresh extracted cells which were heterogenic, therefore their results were in some groups diverse20. Other authors [21,22], and our current experiments confirmed clear stimulating effect of titanium alloy TiO_2 coated on fibroblast proliferation during simultaneous culture, also silicon dioxide surface layers on titanium alloy basis similarly favourably affect fibroblast culture. It is probably related to SiO_2 dissolving in surrounding fluid and direct stimulating effect on fibroblasts – proliferation, protein synthesis. However this process reveal enough intensity with complex layers for instance TiO_2/SiO_2. Pure SiO_2 coatings dissolve too quick, not seldom leading to cytotoxic effect.

In present experiment we did not evaluate or even observe cells on the surface of testing biomaterials. However observing cell activity nearby the implant edge, we noticed that cell density in direct specimen contact is the highest in groups with titanium alloy plates coated TiO_2 and SiO_2 (PHOTO 2 a and 3b). These observation were confirmed in other authors works. Sol – gel titanium dioxide surface layers increased fibroblast's and osteoblast's adhesion, they also increased hydroxyapatite formation, causing the biomaterial more biocompatible [21,23]. It is connected with earlier commented (in introduction) hydrophilic, protein absorbable and cell friendly chemical structure of testing material surface.

Metal implants coated by testing surface layers were designed as bone implants. However effect on contact with skin, gums or other soft tissues is of similar importance. Connection between implant and soft tissues has particular importance in dentistry but also in orthopaedics, trauma surgery (e.g. external fixators) or patients with prolonged vascular access (e.g. dialysed). Very few reports concerning sol - gel TiO_2 and SiO_2 biological properties were carry out on fibroblasts cultures. Our current experiment provided data demonstrating very good tolerance of titanium plates with sol-gel oxide layers on fibroblasts. Therefore we might conclude that implanted biomaterials into soft tissue containing fibroblasts as a basic cell element would also react favourably. There are scientific reports that TiO_2 coated materials comparing to austenitic steel (316L) or non modified surface titanium implants, in vivo, provoke relatively lower local inflammatory response, and cause better implant integration with surrounding tissue – both cellular and non-cellular tissue elements [24].

Many researchers observed that on smooth surfaces cell adhesion is poorer. On the contrary to irregular, rough or porous surfaces, smooth surfaces result in decreased cell proliferation, differentiation and specific function [25,26]. Certainly dynamics of these processes depend on histological cell type. In our present experiment we did not observed influence of roughness on results. The method and project of the experiment unfortunately limited our observation in this matter. Each specimen was located into existing cell culture and we did not observe cell reaction on material surface, only in their surroundings. Therefore we did not evaluate number and strength of adherent cells.

There are lots of method of implant surface modifications. Generally we distinguish them on biochemical modifications – lie on attaching biological active organic compounds, physico – chemical modifications – changing of chemical composition and physical properties like microstructure, surface topography or corrosion resistance. Our tested metal specimens were modified physically and chemically. To improve surface properties we applied sol – gel method, the method becomes more and more popular because it is simple, cheap, allow to form coatings on different shaped or structured materials. The method also let control on chemical composition of the layer, allow enrich it with additional compounds resulting in multicomponent layer. Important features of sol - gel coatings are their mechanical properties; high durable and strong adhesion to basis.

This paper is early report regarding our research on biological properties of new complex biomaterials. Very good results, above all concerning TiO_2 and SiO_2 coatings on titanium alloy implants encourage to attend wider research in vitro, especially influence on bone cells of these materials.

Conclusions

Uncoated austenitic steel plates (316L) did not reveal cytotoxity, but slightly inhibited fibroblasts proliferation. TiO_2 and SiO_2 coated austenitic steel plates clearly inhibited fibroblasts proliferation, did not led to cell necrosis, but their presence unfavourably influence the culture.

Titanium alloy plates TiO_2 coated were inert for fibroblasts – without cytotoxic effect, but no cell proliferation increased. Titanium alloy plates SiO_2 coated and pure titanium alloy (uncoated) created the best environment for fibroblast culture, we obtained increase of fibroblast proliferation, particularly titanium with SiO_2.

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