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Aspects of Bioperformance of Some Polymeric and Metallic Materials Used as Support for Cell Growth and Proliferation

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The paper presents various aspects of thin films performance used as support for cell growth and proliferation. Two kinds of biofilms are investigated: biopolymeric, based on collagen with different synthetic poly-alcohols, and titanium oxide. Polymeric films were obtained using a "casting solution method." The metallic surface was treated in different ways in order to put in evidence effects of chemical and topographical characteristics on cell response. The cytotoxicity test for fibroblast growth using direct contact method, with cultures of human bone tissues was the in vitro biocompatibility evaluation in both cases of polymeric and TiO_2 films.

Keywords: biopolymeric collagen films; titanium oxide

INTRODUCTION

One of the main aspects of bioperformance of implant materials is related to their short, medium and long term stability and integrity in connection with cell growth and proliferation around implant [1]. Biomaterials are developed in the aim of serving to solve clinical problems and testing materials for their biocompatibility, both *in vitro* and *in vivo* conditions, is a way to evaluate implant behavior. The non static aspect of biocompatibility process is related to the possibility for an implant to be osseointegrated today and may be not tomorrow.

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Non perfect integration of the implants into the bone arise problems, as gaps between the implant and the bone open for particles or bacteria, which leads to a chronic inflammatory response and finally to mechanical failure of the implant [2]. Because of this, every year many implants have to be exchanged and this number is expected to grow in the future due to an increasing life cycle. Also host tissues may change in the future as a result of aging or disease. Corrosion and various mechanical aspects as wear and fatigue changing in time are other arguments for understanding biocompatibility as an ongoing phenomenon. At a conceptual level it is to point out the dynamic aspect of biocompatibility, taking into account that like other important scientific notions that change over time, the biocompatibility concept has evolved in conjunction with development of biomaterials devoted to specific medical applications. However the inertness principle reflected in the dictionary of biomaterials as definition of *biocompatibility* like "the quality of not having toxic or injurious effects on biological systems" [3] at the present was extended. According to revisiting original Williams' definition of biocompatibility which still stands regarding an individual material, the discussions could be more specific and move from a "materials base to an applications base" [4]. "Biocompatibility may have to be uniquely defined for each application [5]."

The adhesion of cells to metallic implant can be essential to the implant success and for the prevention of infection. Various procedures to functionalize implant surface were proposed, including thin polymeric films chemically bonded to titanium coupons via silane-glutaraldehyde molecules [6]. The polymer surface should then allow a direct adhesion of cell to close the gap between bone and implant early after implantation. One of the suitable biocompatible polymer coating other than chitosan [6] is collagen [7] and this paper is an approach of biocompatibility performance of thin inorganic and organic films as substrates for cell attachment.

EXPERIMENTAL PART

The tested biofilms materials used in order to build a friendly biointerface were polymeric collagen thin films and TiO_2 oxide on titanium and titanium alloys.

The studied polymeric films based on collagen structure and different synthetic poly-alcohols as poly vinyl alcohol (PVA) and poly glycol (PLG) include structures based on acid collagen hydrolysates (HA), neutral collagen hydrolysates (**HO**) and collagen gel according to

TABLE 1 Type of Collagen Biofilms

Synthetic polymer	Molecular weight of synthetic polymer	Collagen type	Time of hydrolysis (h)	Average molecular weight of HA or HO
A: PVA		**HO_2**	2	72.000
B: PVA		**HO_8**	8	16.600
C: PVA		**HA_4**	4	11.000
D: PVA		**HA_2**	2	15.000
E: PEG I	400	Collagen gel		
F: PEG II	4000	Collagen gel		

PVA–poly(vinilic alcohol); PEG–poly(ethylene glycol).

Table 1. According to this table the average molecular weight of the collagen hydrolysates mainly depends on the hydrolysis time.

The majority of the films [7] were obtained using a "casting solution method." The collagen was stabilized by cross-linking and finllay cross-linking step was achieved by dehydrothermal treatment [8]. The cytotoxicity test for fibroblast growth using direct contact method [9], with cultures of human bone tissues was the *in vitro* biocompatibility evaluation in both cases of polymeric and TiO_2 films. In the direct contact method, secondary cultures of human skin fibroblasts (HSF), were obtained, grown, and subcultured at 37°C in a humidified incubator equilibrated with 5% CO_2. The cells were seeded on the films at a density of 1×10^5 cells/mL and cultured for up to 7 days.

Also in both cases surface analysis type Atomic Force Microscopy (AFM) was performed [8]. Two kind of image analysis were selected as following: AFM images analysis, and Image analyses using Sygma Scan programme able to give information about a single cell or about a selected group of cell.

The physicochemical investigations of new collagen hybrid films were performed using infrared analysis, gel chromatography and viscosimetric method for molecular weight determinations, and porosimetry.

The composition of the metallic samples is the subject of Table 2

Regarding the effect of chemical and topographical characteristics of biomaterials on cell response, investigations were performed using surface treated physico-chemically (chemically polished in 3% HF+ 20% HNO_3 and ultrasonic procedure) or biochemically with collagen gel, taking into account the correlation between surface quality and film stability [10].

TABLE 2 Chemical Composition and Structure of Implant Materials

	% Wt.						
Sample	Al	Fe	V	C	O	N	Ti
Ti	0.005	0.095	–	0.04	0.056	0.045	rest
Ti-5Al-4V	4.88	0.021	3.72	0.048	0.175	0.0153	rest
Ti-6Al-4Fe	6.12	3.87	–	0.18	0.26	0.035	rest

Evaluation of bioperformance involves electrochemical and complementary methods of stability determinations as following:

- monitoring of the variance of electrode potential in open circuit in short, medium and in long term
- cyclic voltametry experiments before and after implantation
- IR (FTIR) spectra for structure determination of by products as a result of adsorption
- Atomic absorption spectroscopy with flame for ion release identification

The experiments were performed in simulated physiological media as Hank, Ringer 1. Ringer 2 and NaCl solutions with a composition listed below:

- Hank: NaCl 8 g/L, $CaCl_2$ 0.14 g/L, KCl 0.4 g/L, $MgCl_2 \cdot 6H_2O$ 0.1g/L, $Na_2HPO_4 \cdot 12H_2O$ 0.06 g/L, $MgSO_4.7H_2O$ 0.06g/L, glucose 1g/L
- Ringer 1: 8.6g/L NaCl; 0.33g/L $CaCl_2$; 3g/L KCl
- Ringer 2: NaCl 0.3 g/L, KCl 0.37 g/L, $NaHCO_3$ 2.44 g/L, $MgCl_2 \cdot 6H_2O$ 0.203 g/L, $MgSO_4 \cdot 7H_2O$ 0.123 g/L, $Na_2HPO_4 \cdot 12H_2O$ 0.07 g/L, and $NaH_2PO_4 \cdot H_2O$ 0.069 g/L.

The temperature measurements were 37°C, the usual temperature of the human body.

RESULTS

Bioartificial polymers blends based on collagen structure was designed taking into account that PEG and PVA could interact with collagen through weak interaction as hydrogen bonds. Structural characterization of samples with various collagens put in evidence the difference between them consisting in the ratio between the intensity of some band. However the infrared spectra are almost similar, with

some differences as a function of collagen hydrolysate and alcohol content: ratio between the intensity of OH hydrogen bonding at $3400\,cm^{-1}$ is different, the intensity being higher for more alcohol and this is an argument for more cross linking due to OH bonded. Regarding values of nNH associated band the initial value is at $3292\,cm^{-1}$ and displacement around $30\,cm^{-1}$ are taking place in all biofilms with collagen hydrolysates.

The three dimensional structure of hybrid biofilms has pores dimension in the 10 nm–2.7μ [9] range depending on collagen and alcohol content and the dependence of average pore radius on collagen is a direct one according to Figure 1.

It is to notice that cytotoxic test indicates a normal phenotype cell culture on the film PVA/collagen hidrolysate and a growth stimulation around 110%. The test indicates important changes in cell proliferation depending on composition, structure and porosity, as it is possible to see in Figure 2 where a modified phenotype cell culture on the bio film PEG/collagen gel is presented. In this case the stimulation growth is 111%.

Regarding TiO_2 films on titanium, as corrosion is an aspect of biocompatibility, the evaluation of its behavior in illiquid is an indication of bioperformance, especially for long term tests and related to experiments done after implantation.

Long-term stability of our implant materials was studied from the monitoring of the open circuit potentials in time simultaneously with pH determinations. As a result of pH changes, potential gradients can appear. Infections can cause variations in the pH from

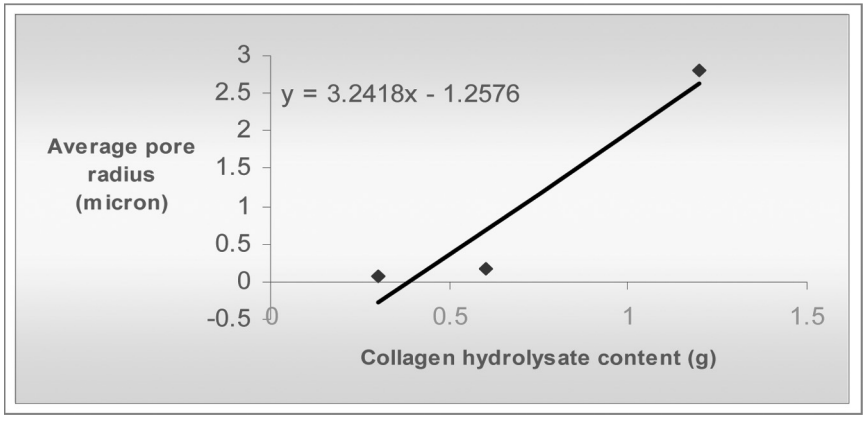

FIGURE 1 The dependence average pore radius and collagen.

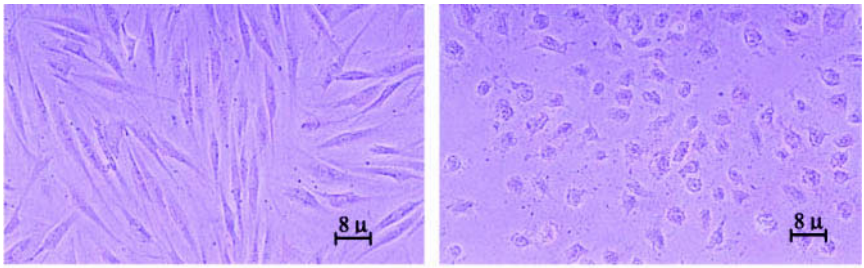

FIGURE 2 Fibroblasts with unmodified (a: control) and modified phenotype (b: PEG/collagen gel film).

4 to 9 and local acidification can arise from the corrosion product reaction.

However, following surgery, the pH can increase to 7.8, decrease to 5, then return to 7.4 within a few weeks, and an implant must resist very well in these conditions.

Variations of open circuit potentials in bioliquids reveal that these potentials are active at the beginning and denote slow dissolution and re-passivation processes on the surface of Ti and its alloys. The tendency for a constant level, denoting passive, protective films is the characteristic of the longer exposure hours [11].

The statistical treatment of the data represented the evolution of potential in time using a regression procedure allows the best approximation of a scatter diagram. In Figure 3 the selected scatter diagram expressed by $Y = -85.08937 - 0.03446X + 8.48464E - 6X$ equation where Y is potential in millivolts and X time in hours is presented.

Monitoring of the open circuit potential and the use of a statistical treatment of the data allow to obtain the scatter diagrams, which are important in the computing of the regression equation and the prognosis of the potential evolution for longer time than the experimental one. Taking into account that the human body is a very complex system, such prognosis should be done with more precautions, but even in such conditions could be a help in bioperformance implant evaluation [10]. The regression equations and the coefficients of determination are presented in the Table 3 as an example, in Ringer 2 solution.

The data from potentiodynamic polarization curves of Ti6Al4V in physiological solutions (Ringer, Hank and 0.9% NaCl) before implantation and after 24 weeks of implantation are shown in Table 4. The corrosion parameters, including corrosion potential (E_{cor},) density

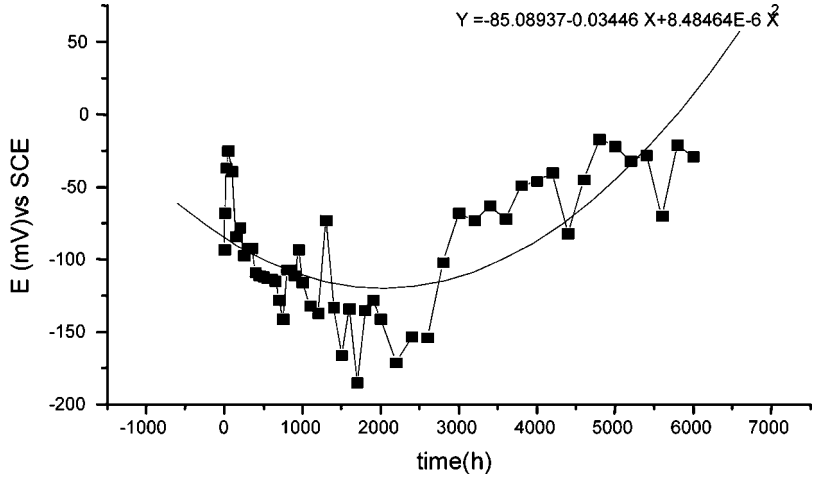

FIGURE 3 The scatter diagram for Ti in Ringer 2 solution.

current (I_{cor},) and passivity current (I_{pas}) obtained from the potentiodynamic polarization test are listed in Table 4 where E_{pr} is the protection potential, and E_{br} the breakdown one. The difference E_{br}–E_{pr} is also calculated, as a measure of corrosion susceptibility, taking into account that if this difference is increasing the corrosion is more aggressive.

Cyclic voltammetry was recorded for TiAlV electrodes in the range covering −0.8 V to 2.5 V. TiAlV shows an anodic current starting at –0.8 V that corresponds to the formation of titanium sub oxides TiOOH and Ti_2O_3. The current peak at about 0.1 V is a consequence of composition change from the Ti (III) to the Ti (IV) oxide, the most stable titanium oxide. With an increasing in the potential, the current density remains almost constant up to around 1.2 V–1.5 V indicate a thickening of anodic surface film. The film is identified as a TiO_2

TABLE 3 Regression Equations

Electrode	pH	Regression equations	Coefficient of determination
Ti	6.98	$Y = -209.9598 + 37.1581\,\lg(x)$	0.569
Ti	2.5	$Y = -85.08937 - 0.03446X + 8.48464E - 6X$	0.604
Ti5Al4V	6.98	$Y = -189.54 + 0.0106x + 0.000001x^2$	0.732
Ti6Al4F	6.98	$Y = -433.9282 + 0.1109x - 0.000002x^2$	0.829

TABLE 4 Electrochemical Parameters from Polarization Curves of TiAlV Samples

Bioliquid	$E_{cor\ (mV)}$	$I_{cor\ (\mu A/cm^2)}$	$I_{pas\ (\mu A/cm^2)}$	$E_{br\ (mV)}$	$E_{pr.}$ (mV)	E_{br}–E_{pr} (mV)
Ringer1[a]	– 250	1.3	12			
Ringer2[a]	– 180	0.01	4.8			
Hank[a]	– 165	0.011	26			
NaCl 0.9%[a]	– 180	3.307	13.6	2946	2356	590
Ringer1[b]	– 537	1.1	11			
Ringer2[b]	– 519	0.02	0.62			
Hank[b]	– 475	0.31	0.68			
NaCl 0.9%[b]	– 545	0.8	18	1325	1050	375

[a]data before surgical intervention.
[b]data 6 months after surgical intervention.

amorphous layer. The breakdown phenomenon is observed only in NaCl solution in both cases, before and after surgical intervention and the potential values were higher than the value that can be reached in a body. It is to notice that potential values are more negative for the samples after intervention, as an argument for the change in performance of biomaterials in the human body, in comparison with simulated situation.

Our spectral data are arguments that the almost immediate event that occurs upon implantation at the interface Ti, TiO_2 / bioelectrolyte is adsorption of various molecules and ions, and such data are supporting other adsorption models existing in literature [12–15]. Hank and Ringer 2 solutions contain phosphate ions and characteristic phosphate band (350 cm^{-1} and 1077 cm^{-1} for bonded phosphate) appears in the IR spectrum of corrosion products; in the same time a decrease of the 3400 cm^{-1} absorbtion band related to the OH of hydrogen bonding is observed.

The stability of films is in direct relation with adhesion for fibroblasts and cell viability, as surface oxide film of titanium with culturing fibroblasts [16].

Figure 4 is an image of fibroblasts grown nearby Ti covered with collagen gel using 2 strata [16]. Viability fibroblasts data were assessed by MTT test, measuring the mitochondrial dehydrogenase activities. The values 100%, 110%, 69.2% are for the control sample, treated Ti sample with 2 strata and with 4 strata of collagen gel, respectively. The decrease in cell viability value for the samples treated with 4 collagen strata is connected probably to the detachment of the film in the case of treatment with more than 2 strata.

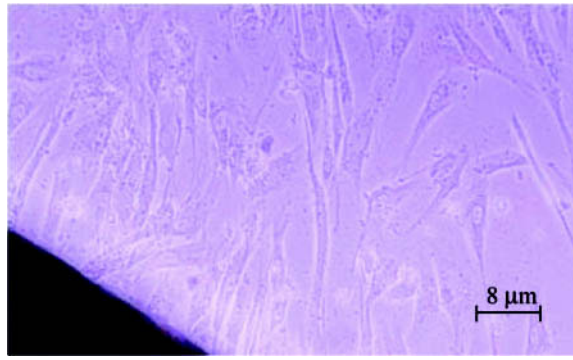

FIGURE 4 Fibroblasts grown nearby Ti covered with 2 stratum of collagen gel.

Figures 5 and 6 are example images for a group of cells and of a representative cell from the selected group of fibroblast grown on Ti covered with collagen gel, and permits biostatistical treatments in order to get quantitative information about cell and cell distribution as it is possible to see in Figure 7, where the statistical procedure was applied and histogram for distribution of cell's diameter was established.

According Figure 7 the majority of cells have diameters between 5 and 10 μm and there are very few with a diameter between 15–20, 20–25 or 25–30 μm long axis size and they represent probably group of 2 or 3 cells.

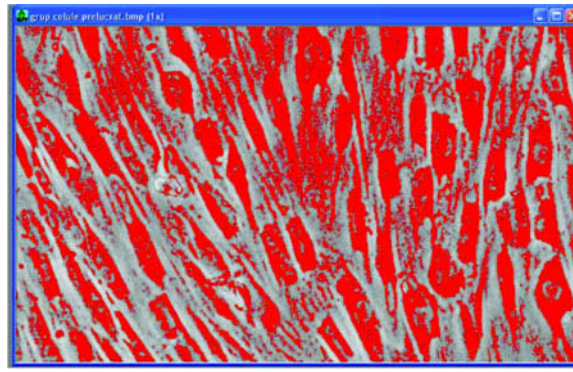

FIGURE 5 Image analysis for a selected group of cells grown on titanium covered with collagen gel.

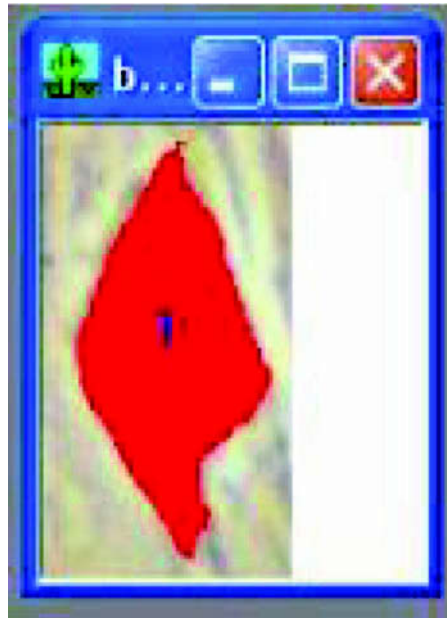

FIGURE 6 Representative cell from the selected group.

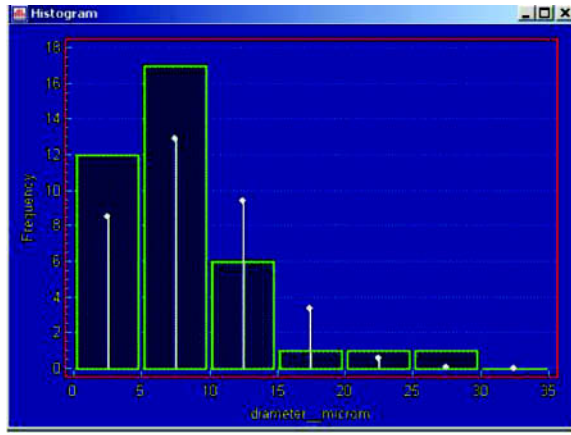

FIGURE 7 Histogram for distribution of cell's diameter.

CONCLUSIONS

1. Inorganic and organic thin films on Ti surface offer a way to produce a better interface biomaterial-living tissue
2. This work provides evidence that cell growth and proliferation should be improved manipulating structure, composition and surface of bio films. The cell viability (assayed by the Trypan Blue exclusion test) is more intense in the case of gel treatment.
3. Electrochemical and biological evaluation of biocompatibility are important aspects in selection materials with high bioperformance and great help in understanding the mechanism at the interface biomaterial living- tissue.

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