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Biocompatibility of aluminium alloys and anodic Al_2O_3

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The purpose of this study is to investigate the biocompatibility, viability and cytotoxicity of ternary aluminium alloys Al-Si-Zr and anodized aluminium EN AW 1050A. There is a wide variety of metals used in different medical devices. For this reason, methods of improving their technical properties and enhancing their biocompatibility are increasingly looked for. Ternary alloys with different contents of the constituents were examined under conditions of co-cultivation with immortalized PDL and serum-freeMcCoy Plovdiv cell cultures. Zirconium was used in the process of casting of the alloys due to its good biological qualities and corrosion resistance. In comparison, the properties of anodized technical aluminium with different oxide film thickness were examined. The process of anodization was carried out in 15% H_2SO_4 for defined periods of time. The results showed that 10 and 20 μm thick oxide films provide better development of the cells compared to the ternary system. Metal surfaces with 10 μm thick oxide film showed the best properties in terms of cells vitality, proliferation and growth.

Keywords: Al-Si-Zr, biocompatibility, cell cultures, anodized aluminium.

INTRODUCTION

Metallic biomaterials are often used to reinforce or replace components of the skeleton, e.g. artificial joints, bone plates, screws, intramedullary nails, spinal fixation, external fixators, valves, stents, dental implants, etc. They should have high tensile strength and fatigue, greater resistance to fracture compared to ceramic materials, etc. The choice of materials for medical implants is also based on properties such as corrosion resistance, biocompatibility, bioadhesion, biofunctionality, genotoxicity, carcinogenicity, cytotoxicity, etc. [1, 2].

There is an enormous variety of materials and alloys used for the manufacture of implants. Some examples are Ti-6Al-4V; Ti-6Al-7Nb; Ti-15Mo; Ti-3Al-2,5V; NiTi; Co-Ni-Cr-Mo-Fe; Ni-Cr; Co-Cr; L-605; zirconium, Al_2O_3 and others. Their production technologies are based on methods of materials science, metallurgy, chemistry and electrochemistry.

Aluminium has good mechanical properties such as lightness, workability, high heat conductivity and electrical conductivity, high corrosion ressistance, etc. However, its low strength necessitates the development of alloys through alloying with other elements. In some cases, the alloying element is zirconium due to its high strengthening effect on the aluminium matrix and its good biological properties [3]. It also facilitates the growth of the inert Al_2O_3 onto the aluminium padding, which defines the corrosion resistance of the native metal. For this reason, the mechanical indicators and the operation of Al-Si-Zr alloys in biological environment were examined.

The use of conventional aluminium as a construction material in medical devices is continuously expanding. There are various methods of treatment to improve its corrosion resistance and biocompatibility. Besides making changes in the chemical composition there are also various surface modification techniques including ion implantation, or deposition of ceramic layers (TiN, DLC, Al_2O_3, ZrO_2), plasma spraying, chemical vapor deposition, physical vapor deposition, etc. Surface modification is the major current area of research in biomaterials [4].

A well-known technique is the obtaining of a protective oxide film on the surface of the metal matrix. Surface oxide film formed on metallic materials plays an important role as an inhibitor of the release of metallic ions, to improve corrosion resistance and tissue compatibility [5]. Al_2O_3 is an inert material with a well-known application mostly in dentistry. The protective effect of Al_2O_3 helps with corrosia and at the same time is capable of reducing the friction on articular surfaces [6]. Aluminium oxide obtained through the process of anodization has the qualities needed for it to be

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more and more widely used in the production of biomaterials. Its porous structure allows it to be filled with a bioactive material, which enhances the compatibility and the antibacterial action of the metal pad [7, 8]

The purpose of this study was to investigate the biocompatibility, viability and cytotoxicity of metal samples of ternary Al-Si-Zr alloys with different amounts of zirconium additions and aluminium (EN AW 1050A) which have been subjected to electrochemical anodization and on which Al_2O_3 layers of various thickness were formed.

EXPERIMENTAL CONDITIONS

The electrochemical experiment was performed in accordance with [9]. It is well known that porous films on the aluminium surface with high pore concentration can be produced by anodic oxidation in electrolytes which dissolve Al_2O_3 (phosphoric acid, oxalic acid, sulfuric acid, etc.). Samples of technical aluminium (EN AW 1050A) with a surface area of 0.02 dm^2 were anodized galvanostatically (15 $mA.dm^{-2}$) at constant temeperature (20ºC) in 15% (w/w) H_2SO_4. The time of Al_2O_3 formation was calculated depending on the desired film thickness [9]. It is also found that the oxides obtained under these conditions exhibit different thickness and the same porosity $\alpha = 0.15$ [10].

Ternary Al–Si–Zr ribbons were prepared from the respective master alloys by flat–flow casting and rapid solidification, as previously described [11, 12]. Samples with a surface area of 0.02 dm^2 were cut and subjected to several biotests.

Cell cultures

PDL cell line of immortalized precursor cells from periodontal ligament.

Cells were cultured in the DMEM/Ham's F-12 1:1 medium with 10 % FCS, 100 I. U. penicillin and 100 µg/ml streptomycin. The procedures for cell culturing and storing were performed according to [13].

Serum-free cell line McCoy-Plovdiv.

Cells were cultured in the DMEM/Ham's F-12 1:1 medium with 15 mM HEPES, 100 I. U. penicillin and 100 µg/ml streptomycin. The procedures for cell culturing and storing were performed according to [14].

Experimental design

Biocompatibility testing

Metal lamellas were placed in 8-well slides with coverslip for cell with a lid for cell cultures. Cells with different innitial seeding density were added and incubated for 96 and 198 h . The state of the cell monolayer and cell morphology cultures was inspected every 24 h using an inverted light microscope (Nikon Eclipse TS100). Assessment of changes in the cell layer was made at the border delineated by the edge of lamella and the slide. Direct visualization of cultured cells was not possible because of the opaque nature of metallic lamellas. Microscopic images were captured using a Nikon camera and a photo documentation system. At the end of the incubation period, the cultures were examined for vitality with the reactant WST-1. The state of the cultured cells onto the metal surfaces was also visualised by a LIVE/DEAD test.

Cytotoxicity testing

In this test McCoy Plovdiv cells were treated with the DMEM/Ham's F-12 1:1 medium pre-incubated with the Al-Si-Zr ribbons for 24, 48, 72 and 96 h . After 24-h of treatment the cytotoxic effect was determined microscopically and by a vitality test using the reagent WST-1. The results were statistically analyzed.

Viability testing

In our measurements we used the reagent WST-1 (Roche Diagnostics, Mannheim, Germany), which was reduced from cellular enzymes (mitochondrial dehydrogenases) to a water-soluble product. Accumulation of formazan product formed correlates directly with the count of metabolically active cells in the culture. The cells were incubated for 4 h with the reagent WST-1 at 37°C, after which the absorbance of the resulting colored product was measured at 450 nm wavelength using the ELISA reader *Sunrise* (Tecan, Maennedorf, Switzerland).

Immunofluorescense

The samples were washed twice with PBS tempered at 37ºC. Cells were fixed with 4% paraformaldehyde/PBS for 10 min and permeabilized with ice-cold acetone for 5 min. Incubation in 3% FCS/PBS for 30 min was performed to block non-specific binding. FITC-conjugated phalloidin (1:40 in PBS) (Invitrogen) was used to stain actin and Hoechst 33342 (3 µg/ml) (Sigma) was used for nuclear staining.

The microscopic examination was conducted by the use of epifluorescence microscope Nikon Eclipse TS100.

EXPERIMENTAL RESULTS AND DISCUSSION

The metal plates subjected to bio testing had dimensions of 1 × 1 × 0.5 mm. They were numbered from 1 to 6, and had the following characteristics of surface treatment:

Table 1. Characteristics of surface treatment

Sample number	Type of treatment
1	untreated aluminum
2	anodized aluminum, oxide film thickness d = 10 μm
3	anodized aluminum, oxide film thickness d = 20 μm
4	anodized aluminum, oxide film thickness d = 30 μm
5	Al-Si-Zr ribbon (w_{Zr} = 0.27 %)
6	Al-Si-Zr ribbon (w_{Zr} = 1.09 %)

Ternary alloy Al-Si-Zr

With a view to studying the application of the new ternary alloys in biological environment, a series of experiments with serum-free McCoy Plovdiv and PDL cell lines of immortalized cells, precursors of periodontal ligament were conducted. 24 h after the start of incubation of McCoy-Plovdiv cells with metal lamellas, in the samples, dead cells, rounded and clustered cells, without well-formed monolayer, were seen near the edges of lamellas. By increasing the time the amount of dead cells was much greater (Fig. 1)

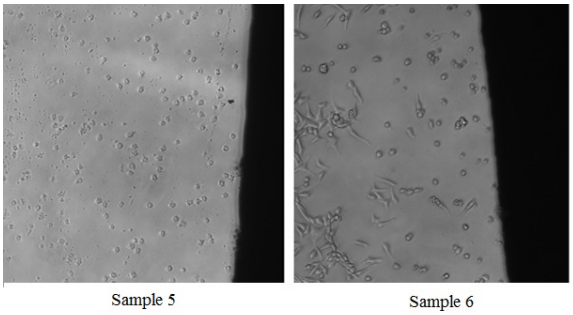

Fig. 1. McCoy-Plovdiv cells cultured with samples 5 and 6 for 96 h

In the cytotoxicity testing in sample 5, reduction in cell count by more than 50% under the influence of the medium in which the lamella stayed for only 24 h was recorded. In sample 6, reduction in cell count by about 30% was recorded.

By increasing the time for which the lamellas stayed in the medium, a trend for increase in the toxicity of these media was established. This was convincingly confirmed by the very low levels of surviving cells in sample 5 (6.5%) and 6 (13.7%), which coincides with our visual microscopic observations. Consequently, four times higher zirconium content in the aluminium alloy increases twice the percentage of surviving cells.

Impairment of cell monolayer, rounded and dead cells near the edge of the lamella were found in samples 5 and 6. The state of the cell layer was assessed at the border delineated by the edge of lamella and the slide using an optical microscope. It was not possible to observe the cells directly in the individual stages of the experiment because of the dense nature of the metal lamellas.

For assessment of the biocompatibility with PDL cells of the different lamellas, at the end of the incubation period they were fixed and stained with fluorescent dyes that allow visualizing the cells adhered to metallic surface. Data from these observations are presented in Fig. 2.

No viable cells connected to the surface could be seen on sample 5. There were fragments of cells and cell nuclei debris. In sample 6, there were few connected cells which were not well spread as a layer or were clustered. The two samples, 5 and 6, had a markedly negative effect on cell growth in *in vitro* conditions. Cell adhesion, spreading of cells, cell proliferation and formation of a monolayer on these lamellas was impeded. Besides the influence of the direct contact with the metal surfaces, an indirect cytotoxic activity of molecules or compounds released/produced in the medium was observed. This toxic activity increases with the time in which metal plates 5 and 6 were incubated in a medium without cells.

Aluminium EN AW 1050A

To examine the effect of different surface modifications on cell growth and biocompatibility, PDL cells were placed with a low initial seeding density. This made it possible to assess the development of the culture and the increase in the number of cells, cell proliferation, adaptation of cells and their colonization on the respective substrate.

Two initial seeding density levels were chosen, for which previous information shows that they reach subconfluent and confluent state, when they are grown onto a glass (the bottom of the axilla) for 4 and 7 days respectively. This could be seen clearly on Fig. 3 (A-1; A-2) and Fig. 4.

Fig. 5 presents the results obtained for the number of PDL cells cultured for 96 and 168 h together with samples 1, 2, 3 and 4. It can be seen that for sample 2 (oxide film thickness of 10 μm) and sample 3 (oxide film thickness of 20 μm) the values are higher (sample 2 - 68.3% at 96 h and 72.6% at 168 h; sample 3 - 52.6% at 96 h and 59.4% at 168 h) compared to those for sample 1 - untreated aluminium (42.8% - 96 h, 45.9% - 168 h).

The absence of toxic effect was evaluated by microscopic observation of native cultures (Fig. 3).

Outside the metal lamellas, the formation of the cell layer consisting of cells with normal morphology and splitting cells can easily be seen. Fluorescent-microscopic images showing the development of cells on the surface of the different samples are shown on Fig. 6.

The best cell development (more living cells adhered to the surface) is seen with samples 2 and 3, and it is weaker (with less living cells on the surface) with sample 4 (thickness of the oxide film 30 μm).

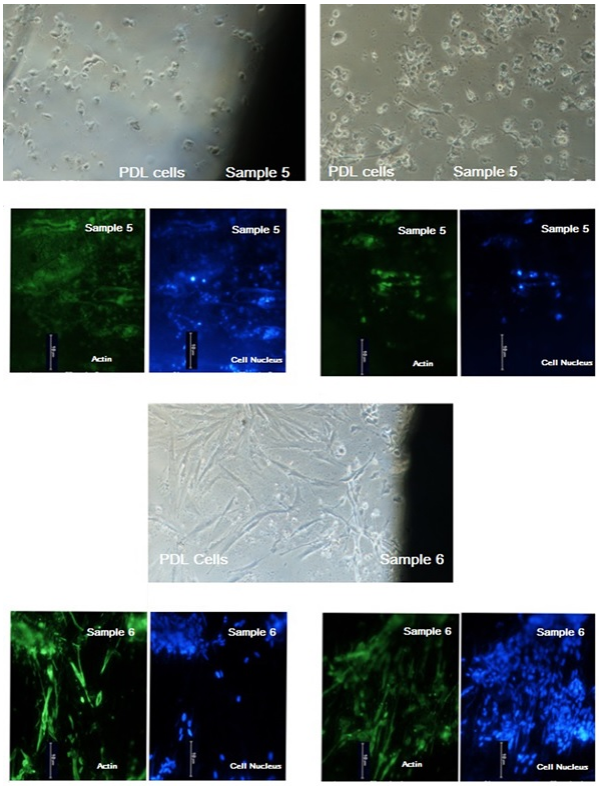

Fig. 2. PDL cells cultured with samples 5 and 6 for 96 h.

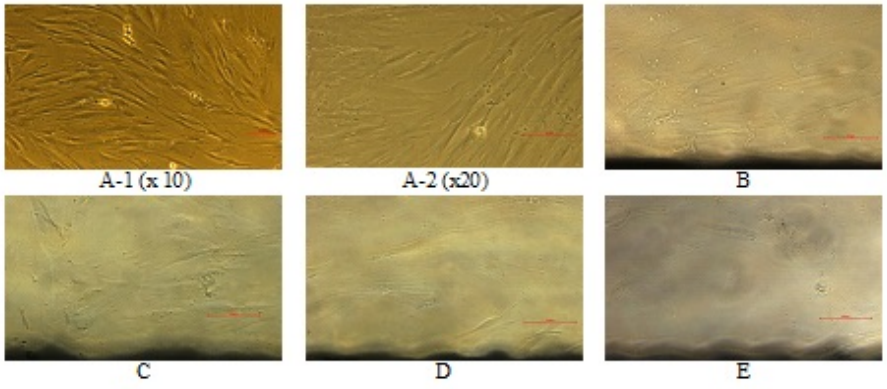

Fig. 3. Human PDL cells, co-cultivated with sample 1(B), sample 2(C), sample 3(D), sample 4(E) for 24h. Control (A) - cells, cultivated on glass (A-1, magnification ×10; A-2, magnification ×20). Inverted light microscope. Magnification ×20.

Fig. 4. Human PDL cells, cultivated on glass for 24h. Visualisation with a reactant LIVE/DEAD and Hoechst 33342. Fluorescence microscope. Magnification ×10.

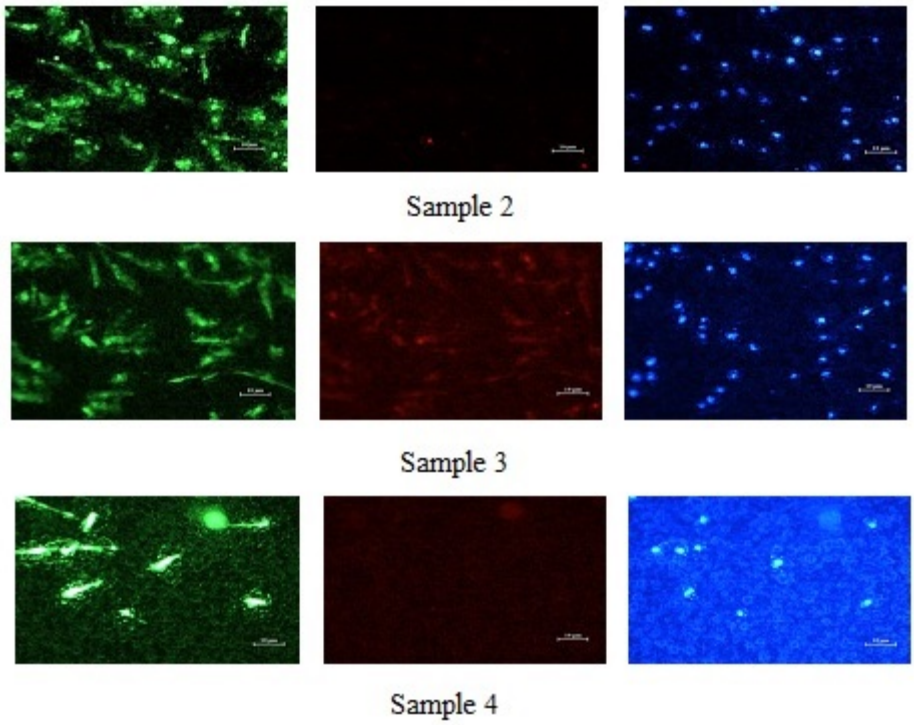

Fig 6. Human PDL cells, cultivated with sample 2, 3, 4 for 24h. Visualisation with reactant LIVE/DEAD and Hoechst 33342. Fluorescence microscope. Magnification ×10.

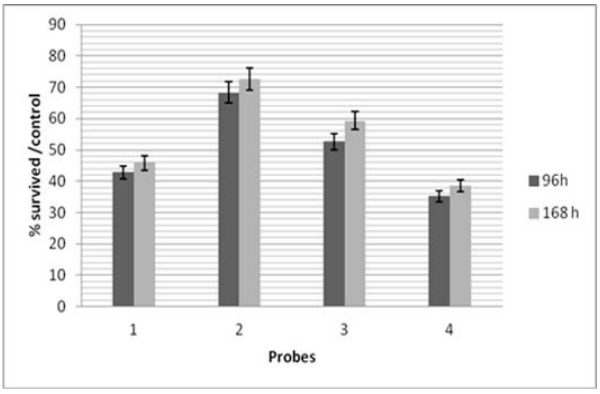

Fig. 5. Viability of PDL cells co-cultured with samples 1, 2, 3 and 4 for 96 h (black bars) and 168 h (grey bars).

CONCLUSIONS

From the experiments and analysis of the obtained results we can make several conclusions.

Al-Si-Zr ribbons - samples 5 (w_{Zr} = 0.27 %) and 6 (w_{Zr} = 1.09 %) impede cell adhesion and cell growth of McCoy-Plovdiv and PDL cell lines. They have a negative impact on the development of McCoy-Plovdiv and PDL cells in conditions of co-culture, initiating changes in cell morphology and cell monolayer and causing cell death. Samples 5 and 6 generate cytotoxic activity in the culture medium, causing cell death of McCoy-Plovdiv cells. The higher content of zirconium in the ternary alloy increases the percentage of surviving cells and improves the biocompatibility of the material.

The experiments with human PDL cells for assessment of the viability and biocompatibility of the investigated samples confirm that the anodic Al_2O_3 films provide better conditions for development of PDL cells compared to untreated aluminium and ternary Al-Si-Zr alloys. Metal surfaces with 10 μm thick oxide film (sample 2) show the best properties in terms of cells vitality, proliferation and growth compared to sample 3 (20 μm thick oxide film) and sample 4 (30 μm thick oxide film).

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БИОСЪВМЕСТИМОСТ НА АЛУМИНИЕВИ СПЛАВИ И АНОДЕН Al_2O_3

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(Резюме)

Целта на настоящата работа е да се изследват биосъвместимост, виталност и цитотоксичност на тройни алуминиеви сплави Al-Si-Zr и анодиран технически алуминий EN AW 1050A. Съществува голямо разнообразие от метали, използвани в различни медицински устройства. Поради това все по-често се търсят методи за оптимизиране на техните механични свойства, а също и за повишаване на биосъвместимостта им. Тройни сплави с различно съдържание на легиращ елемент цирконий са изследвани в условия на съвместно култивиране с клетъчни линии PDL и McCoy-Plovdiv. Цирконият е влаган в процеса на леене на сплавите поради добрите му биологични качества и корозионна устойчивост. За сравнение са разгледани свойствата на образци от получен по електрохимичен път оксиден филм с различна дебелина върху технически алуминий. Анодирането е проведено в разтвор на 15% H_2SO_4 за определени интервали от време. Резултатите показват, че оксидни филми с дебелина 10 и 20 μm осигуряват по-добро клетъчно развитие в сравнение с тройната система Al-Si-Zr. Оксиден филм с дебелина 10 μm демонстрира най-добри качества по отношение на клетъчна виталност, пролиферация и растеж.