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A Study on the Evaluation of the Cytotoxicity of Al_2O_3, Nb_2O_5, Ta_2O_5, TiO_2 and ZrO_2

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Abstract

Nearly all metals used in implants are pure or alloys composed of transition elements forming spontaneously passive layers on the surface in electrolytes in a wide pH region. The biocompatibility of these metals used in implants is tested before surgical implantation. Determining the biocompatibility of these metals is implemented firstly by the testing of their cytotoxicity. The first step consists of evaluating the cell morphology and proliferation. In this study, glass slices were coated with Al_2O_3, Nb_2O_5, Ta_2O_5, TiO_2 and ZrO_2 using the sol-gel technique. Then Vero fibroblast cells were inoculated with these slices and the influence of metal oxides on cell proliferation and morphology was observed. Metal oxides did not affect the cell morphology. However, the cell counts varied compared to those of the uncoated glass slices (control): cell numbers decreased 30% with Al_2O_3 and Nb_2O_5, by 45% with $Ta_2O_5$and by 58% with ZrO_2. There was no change in cell numbers with TiO_2. These results might give an idea about the cytotoxicity of the metal oxides tested. However, the application of other cytotoxicity tests and the testing of the samples with several other cell types seem to be necessary.

Key words: Cytotoxicity, Cell morphology, Oxide layer, Sol-gel coating, Glass.

Introduction

The biological properties of biomaterials are dominated by their chemical, physical and physicochemical properties. While the behaviour of ceramics is bioinert or even bioactive (hydroxyapatite), metallic materials have extremely different biological properties.

Nearly all metals used in implants are pure or alloys composed of transition elements forming spontaneously passive layers on the surface in electrolytes in a wide pH region. Concerning organic or inorganic reactions taking place at the implant/body system, those primary corrosion products (mainly oxides and/or hydroxides) lying on the surface are responsible to a great extent for the biocompatibility of the implanted metal. This is because these layers possess large surfaces that may interact with either the tissue or body fluid (Breme and Helsen, 1998; Thull, 1998).

One criterion for the suitability of materials as a biomaterial for close tissue contact is the physicochemical reactivity of the surface. Bulks and surfaces have properties as a metal, a semiconductor or an insulator. The differences are caused by the electronic structure of the materials described by the density of electronic states and their occupation by charge carriers.

Some materials in dental and orthopaedic implants, in particular spontaneously passivating met-

als of groups 4b and 5b of the periodic table elements, show a close connection to the force-transmitting tissue and are virtually free of reactions with recognition cells in the immunological system. This behaviour, sometimes referred to as integration, is based on the oxide layer arising spontaneously on the surfaces of passivating metals within the extracellular fluid. Integration is an important source of biocompatibility. Causes of tissue reactions around alloplastic materials are identified as cell-mediated hypersensitivity to an implant component and tissue modifications due to the presence of wear particles, debris and corrosion products from the prostheses used.

The interaction between the surface of the implant and the body's electrolytes begins with the adsorption of charged ions and polarisable molecules, such as water molecules or biological macromolecules. Through forces of attraction and repulsion and the exchange of charge carriers, as electrons for metals or electrons or holes for semiconducting coatings, reactive and reversible or irreversible structural changes to adsorbed substances can take place. In particular, in organic macromolecules, intramolecular and intermolecular bonds, together with oxygen bridge bonds, may break down, giving rise to structural or conformational changes, or both. Conformational changes may arise as a result of an exchange of charge carriers between the surface of the biomaterial and the biological macromolecules.

The nature of the interaction between the implant/body system depends on the physical, chemical and physicochemical properties of the implant material, such as the heat of formation, the solubility product and the dielectric constant. The more negative the value of heat of formation of the primary corrosion products of oxides and hydroxides developed on the surface of the implants compared to that of water as demonstrated by the oxides and hydroxides of Al, Cr, Nb, Ti, Ta, V and Zr, the more stable the structure thermodynamically and, therefore, the less probable the interaction between the oxide or hydroxide and the body fluid. Consequently, thermodynamically stable corrosion products have a low solubility product and a low solubility in the body fluid. Thermodynamically stable primary corrosion products with a low solubility in body fluid are in stable equilibrium with only a low reactivity with the proteins of the surrounding tissue. Ti, Ta and Nb are reported to be biocompatible because they form protective surface layers of semiconductive or non-conducting oxides. Because of their isolating effect, these oxides are able to prevent to a great extent an exchange of electrons and therefore a flow of ions through the tissue (Zettner *et al.*, 1980). This isolating effect may be demonstrated by the dielectric constants of different metal oxides. There are 3 groups of oxides; while TiO_2 (rutile), Fe_2O_3 and Nb_2O_5 have constants even higher than that of water, Al_2O_3, Cr_2O_3, Ta_2O_5 and ZrO_2(Fink and Baty, 2000) have a lower isolating effect and a higher conductivity. For oxides of Ni and V, dielectric constants are not available because of their high conductivity.

The biocompatibility of those metals used in implants is tested before surgical implantation. Biocompatibility testing includes numerous methods starting with mechanical, physiochemical and electrochemical investigations, i.e. corrosion tests from in vitro and in vivo tests such as implantation in animals and preclinical evaluation in humans to final clinical use in patients. Cytocompatibility is the in vitro adequate behaviour of cells in the presence of biomaterials, whereas cytotoxicity is the harmful or noxious unwanted effect induced by a biomaterial in cell-culture systems.

The cytotoxicity of biomaterials must be studied in vitro in early stages before animal experiments in order to discard highly cytotoxic candidate materials and to reduce the amount of in vivo testing to a minimum. The complexity of the in vivo situation can never be simulated in vitro (ISOL/TR 7405 (F) Standard, 1984) and further experiments should be considered in order to allow a conclusion to be drawn: cytocompatibility tests (Breme, 1998) involving cells in direct contact with material samples, and in vivo toxicity tests.

The purpose of this study was to establish whether an oxide layer is able to protect cells from toxicity in metallic biomaterials like dental implants, coronary stents or prostheses. For this purpose, metal oxides (Al_2O_3, Nb_2O_5, Ta_2O_5, $TiO_2$and ZrO_2) coated on glass platelets were tested by in vitro cytotoxicity tests according to ISO 10993-5 (1992) standards (Kirckpatrick *et al.*, 1998) in order to determine their effect on cell-culture systems, which is one of the main components of biocompatibility.

Materials and Methods

Production of metal oxide layers on glass surfaces

The substratum is of circular glass platelets 13 mm in diameter and 4 mm thick coated with 5 different metal oxides,(Al_2O_3, Nb_2O_5, Ta_2O_5, TiO_2 and ZrO_2) using the sol-gel coating technique. If the oxide layers are of 100 nm or above, the biocompatibility is not influenced by the substratum and the results represent the biocompatibility of the oxide coating only (Velten *et al.*, 2002).

In order to perform the sol-gel process, mixtures of a metal alkoxide (tetrabutyl orthometal MeBOT, $Me(OC_4H_9)_4$) and a solvent ethanol, C_2H_5OH, are used (here, Me stands for the metal element). Using this sol, the glass substratums are coated through spin-coating (6000 rpm). The coating is repeated 2-4 times in order to be able to obtain coating thicknesses of minimum 100 nm. In total 9 specimens per oxide coating were produced. Following the spin-coating, the specimens were subjected to drying at 130 °C for 30 min. During this drying, most of the ethanol, butanol and water evaporate, resulting in an agglomeration of the MeO particles in the film (gel formation). Finally, the specimens were annealed at 500 °C for 1 h to accomplish the crystallisation and solidification of the amorphous MeO surface.

When all the metal oxide layer coating was finished, all glass platelets were cleaned by ultrasonic cleaning preceding 15 min waiting in 99% ethanol. Then all platelets were sterilised by autoclaving for 15 min at 120 °C before cell culturing.

Cell culture

In vitro cell culture experiments were carried out with African green monkey kidney cells (Vero fibroblasts) cultured on the samples for 7 days. The cultivation medium consisted of RPMI 1640 supplemented with 10% foetal calf serum, L-glutamine (2 mM ml^{-1}), amphotericin and gentamicine.

Counting of the cells

The cells were detached from the flasks using trypsin-EDTA. One millilitre of the trypsinated cell suspension (20 μl) was transferred immediately to the edge of a Fuchs-Rosenthal chamber (Brand, Germany, Code: 719806). Then the chamber was placed under a light microscope (Nikon, inverted)(X10) in order to count the cells.

The cell numbers were also examined in the presence of metal oxides. The Vero fibroblast cells were seeded onto the surface of samples in 12-well plates. The seeding concentration was measured (cells per well). The glass samples coated with 5 different metal oxides (Al_2O_3, Nb_2O_5, Ta_2O_5, TiO_2 and ZrO_2) were also placed 1 per well with their coated sides facing upwards. Uncoated glasses were placed into one of the wells as a reference platelet as well. An equal amount of medium (2 ml) was distributed into each well. This was followed by the incubation of the plates using the procedure mentioned above. After 7 days of incubation, the samples were placed in new plates, and the cells on the samples were subsequently trypsinated (100 μl/well). Trypsinated cell suspensions over the glass samples were transferred to the Fuchs-Rosenthal chamber. Thereafter, the cells were counted 3 times for each metal oxide sample. The average of all 3 cell counts was considered the actual cell number.

Investigating the cell morphology

The cell morphology on metal oxide coated samples was investigated through staining the platelets with May-Grünwald solution. Subsequently, the cell morphology was investigated under the light microscope. The cell form, the size and the form of the nucleus, ectoplasm, endoplasm, cell spreading, granulation and vacuoles, the lenght of the laminopodium and the presence of any sign of apoptosis, necrosis and cell lysis were investigated.

Results and Discussion

The preliminary results of the tests concerning the biocompatibility of metal oxides, namely cell proliferation and cell morphology, are reported in the following. The influence of metal oxides (Al_2O_3, Nb_2O_5, TiO_2, Ta_2O_5, ZrO_2) on cell morphology is shown in Figure 1 along with uncoated glass (control) as a reference. The cell morphology of the metal oxide coated glasses is the same as that of the uncoated glass samples' surfaces. In addition, when the cell morphology of the 5 different metal oxides is compared, it is found that the cell morphology is not altered with respect to the metal oxide type. The nucleus could be observed and the nucleus/cytoplasma ratio was normal in all samples. All cells were intact and showed fibroblasts with a typical spread.

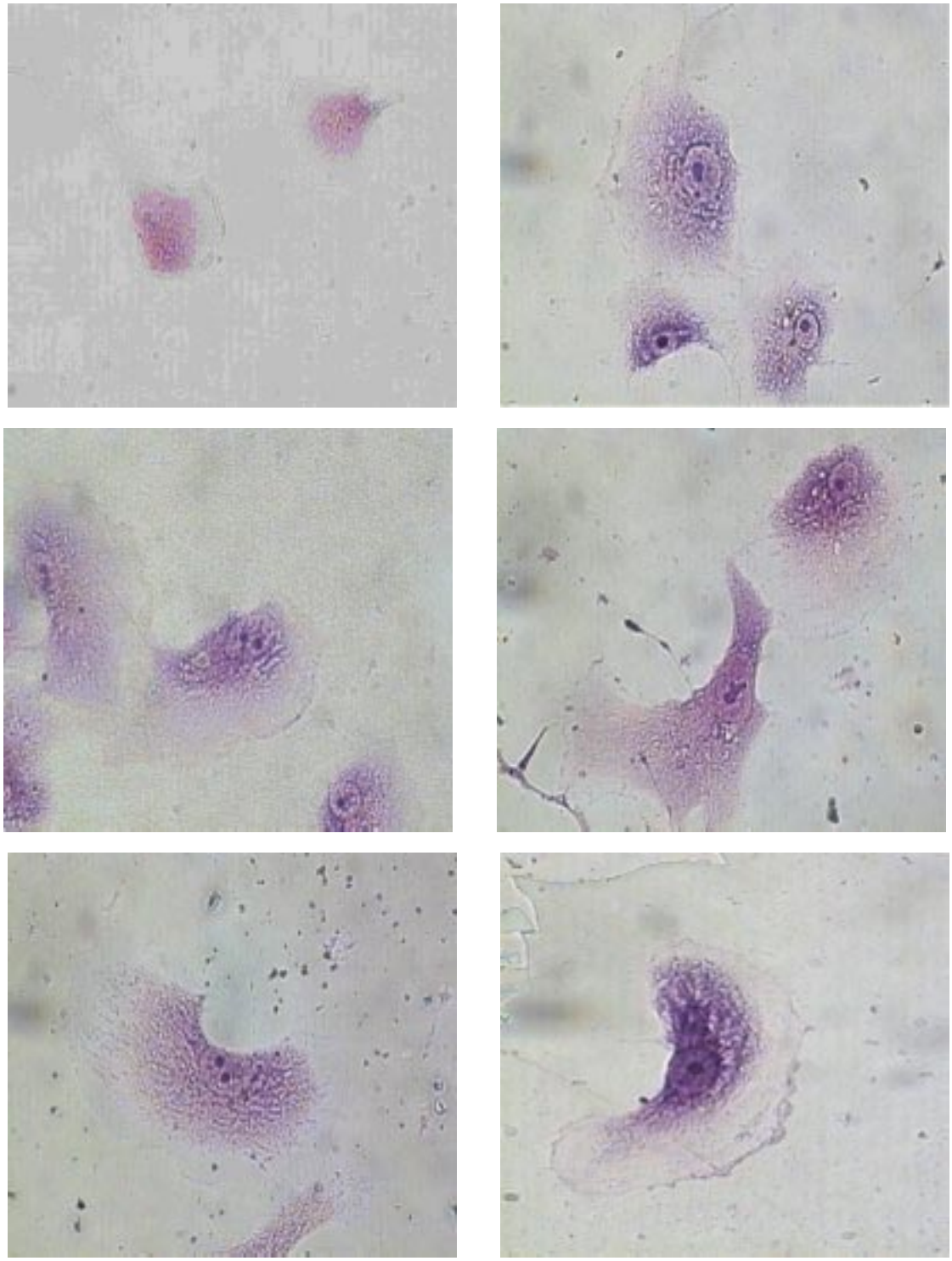

Figure 1. The microscopic appearance of fibroblastic cells on glass surfaces coated with 5 different metal oxides after incubation and staining with May-Grünwald solution. The cells are intact and there are no signs of cell lysis or apoptosis. The nucleus and the laminopodium of the fibroblastic cells are prominent under an inverted microscope (please note that the pictures are taken via a digital system attached to the microscope unit).

The effect of metal oxides on cell proliferation is investigated by counting the cells attached to the glass surfaces after incubation (Figure 2). The mean cell counts vary when compared to uncoated glass platelets (control, n = 62): cell numbers decrease by 30% with Al_2O_3 and Nb_2O_5;by 45% with $Ta_2O_5$and by 58% with ZrO_2. The cell numbers of TiO_2 (n = 63) are almost the same as those of the control, whereas the cell numbers of ZrO_2 are the lowest (n = 26). These observations, except for the Nb_2O_5, are reasonably in accordance with the dielectric constants range reported with the respective oxides examined.

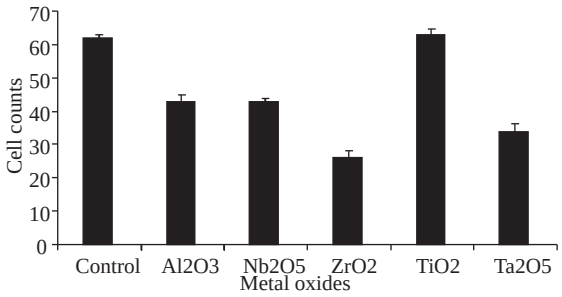

Figure 2. The effect of metal oxides on cell proliferation. The control well contained uncoated glass platelets, the medium plus Vero cells, whereas the others contained additional glass platelets coated with metal oxides.

Titanium and titanium based alloys are increasingly being used for surgical implants in cardiovascular, orthopaedic, maxillofacial, otological and visceral surgery. Titanium based alloys contain low amounts of other metals such as alumina, vanadium, niobium, tantalum, manganase and zirconium (Wadowitz and Breme, 1989). The sound biocompatibility of titanium and its alloys is reported to be (Wilson and Hench, 1994; Hench and Wilson, 1993; LeGeros, 1993; Ratner, 1993) due to a thin but adherent layer of native oxides, which is spontaneously formed on its surface. The presence of an oxide layer on the surface apparently plays an important role in the favourable tissue response to titanium based implants (Eisenbarth *et al.*, 2002). A good level of biocompatibility exhibiting no cytotoxicity was also detected in studies with ZrO_2 and Al_2O_3(Dion *et al.*, 1994; Kim *et al.*, 2000; Toricelli *et al.*, 2001). In addition, the biocompatibility of zirconia and alumina as a joint surface material for artificial joints was shown (Warashina *et al.*, 2003). However, the superiority of titanium based implants to other metallic implant materials has been certified in many clinical applications.

In this study, the oxides of metals used in titanium alloys, i.e. alumina, zirconia, tantalum and niobium as well as titanium, were tested in terms of cytotoxicity. The cell morphology was not affected with respect to the oxide type. However, the cell counts varied from one oxide to another. The low cell counts obtained with Al_2O_3, $Nb_2O_5$and Ta_2O_5 and the least counts with ZrO_2 do not mean that those metal oxides are cytotoxic. However, the good results determined with TiO_2 in both morphology and proliferation tests in this study indicate that TiO_2 is probably the best metal oxide to be used for coatings with the sol-gel technique.

All these results might give an idea about the cytotoxicity of the metals tested. A definite conclusion should be drawn after the application of other cytotoxicity tests (cell adhesion tests, inflammatory tests, gene sequencing etc.) that are necessary to fully cover the biocompatibility. Commercial kits detecting in vitro cytotoxicity (neutralised red test, MTT etc.) are also available. In addition, the results should also be verified by in vivo animal experiments.

In conclusion, cytotoxicity tests are the first step of biocompatibility tests. Currently, studies on these tests to improve the biocompatibility of biomaterials are increasing. The demand for such kinds of biomaterials would be expected to rise in rapidly developing Turkey. In order to meet this demand, more work on improvements of the biocompatibility of biomaterials is needed. The experience gained in this study would be very useful for biomaterial testing studies planned in our university and the application of biocompatibility tests would allow the testing of cell-biomaterial interactions in Turkey.

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