Evaluation techniques of metallic biomaterials in vitro

Takao Hanawa*

Biomaterials Research Team, National Research Institute for Metals, 1-2-1 Sengen, Tsukuba 305-0047, Japan

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Abstract

Metals and alloys are widely used as biomedical materials and are important in medicine and they cannot be replaced with ceramics or polymers at present mainly because of their high strength and toughness. Since safety is the most important property of biomaterials, corrosion-resistant materials such as stainless steel, Co–Cr–Mo alloy, commercially pure titanium, and titanium alloys are employed as biomaterials. Evaluation techniques for corrosion with culturing cells, the characterization of reconstruction of surface oxide film, fretting fatigue, cytotoxicity, and biocompatibility are reviewed in this paper. These techniques are original and characteristics in the field of biomaterials that should contribute to the proper evaluations of biomaterials in vitro.

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1. Introduction

The demand for metallic materials in medical and dental devices is large. Metals and alloys are widely used as biomedical materials and are indispensable in the medical field. In particular, toughness, elasticity, rigidity, and electrical conductivity are essential properties for metallic materials used in medical devices. Conventionally, metallic materials are essential for orthopedic implants, bone fixators, artificial joints, external fixators, etc. since they can substitute for the function of hard tissues in orthopedics. Stents and stent grafts are placed at stenotic blood vessels for dilatation. Therefore, elasticity for expansion and rigidity for maintaining dilatation are required for the devices. In dentistry, metal is used for restorations, orthodontic wires, and dental implants.

For mechanical reliability, metallic materials must be used and cannot be replaced with ceramics or polymers. The most important property of biomaterials is safety. Therefore, corrosion-resistant materials such as stainless steel, Co–Cr–Mo alloy, metallic titanium, and titanium alloys are employed. In particular, the Co–Cr–Mo alloy is used under conditions of wear because of its high wear resistance. Noble-metal-based alloys, such as gold alloys and silver alloys, are used in dentistry.

Proper techniques must be selected to precisely evaluate metallic biomaterials in vitro and many efforts have been made to develop the evaluation techniques. In this paper, the evaluation techniques mainly in vitro of metallic biomaterials newly developed in our research group are reviewed. This paper also explains the significance of these developments.

2. Corrosion

The concentrations of chloride ions in serum and interstitial fluid are 113 and 117 mEq l⁻¹, respectively, which is about 1/3 the concentration of brine and a seriously corrosive environment for metallic materials. Body fluids contain various amino acids and proteins that influence metallic corrosion [1] because they are electrolytes. In addition, the concentration of dissolved oxygen is 1/4 that of air in venous blood and 1/80–1/4 that of air in intercellular spaces [2], which accelerates the corrosion of metallic materials. Changes in the pH of body fluids are small because the fluids are buffered solutions and the pH usually remains between 7.0 and 7.35 [2]. The pH of the hard tissue into which a material is implanted decreases to approximately 5.2 and then recovers to 7.4 within two weeks [3]. The cell is also a kind of charging body that may influence the corrosion of metallic materials.

Metallic materials themselves do not show any toxicity, but some dissolved metal ions, corrosion products, and wear
debris may show toxicity when they combine with biomolecules and cells. Corrosion and electrochemical properties of metallic materials in biological environments are reviewed elsewhere [1].

2.1. Corrosion with macrophage [4]

When a material is implanted, it is recognized as a foreign body by immunological processes and macrophages (Mφs) adhere to the surface of the material. Mφ generates active oxygen species without response to particles that can be phagocytosed. Mφ produces much more active oxygen species when they phagocytose particles. O$_2^-$, one of active oxygen species, initiates and propagates free-radical chain reactions. An intracellular dismutation of O$_2^-$ catalyzed by superoxide dismutase produces H$_2$O$_2$, which has a much longer lifetime and higher permeability against cell membrane than O$_2^-$. H$_2$O$_2$ reaches the surface where Mφ has adhered. The metal surface is hyperoxidized by H$_2$O$_2$ that may induce the corrosion of metallic materials.

To determine the effects of Mφ on metal ion dissolution, titanium disks were immersed in different solutions and titanium ions dissolved from titanium disks into each solution were quantified. The results revealed that active oxygen species generated by Mφ induced metal ion dissolution as shown in Fig. 1. In particular, the ion dissolution was accelerated with high-density polyethylene (HDP) because Mφ which phagocytosed HDP generates more active oxygen species than Mφ which did not phagocytose any HDP. In addition, antioxidant enzyme decreases the dissolution of titanium because of the decrease of active oxygen species. These are some of the causes for metal ion dissolution from titanium implants in the absence of wear and fretting in vivo.

2.2. Electrochemical analysis with cultured cell [5]

The corrosion behavior and biocompatibility of metallic biomaterials must be evaluated under culturing cells in order to understand the properties of materials in vivo. Electrochemical analyses are useful to characterize the corrosion behavior and the interface structure of metal and solution, in situ. Therefore, a new electrolytic cell with which both the electrochemical measurement and the cell culture can be performed simultaneously has been developed. The developed electrolytic cell is shown in Fig. 2. The cell can be sterilized in a saturated water vapor at 393 K. The culturing cells attach to only the working electrode (specimen) surface.

Using the newly developed instrument, open circuit potential ($E_{open}$) of titanium with L929 fibroblasts incubated in cell culture medium (MEM) plus fetal bovine serum (FBS), in MEM + FBS, and in MEM, was measured for 30 min, then AC impedance was measured with 5 mV amplitude in a frequency range from $10^5$ to $10^{-3}$ Hz at $E_{open}$. Bode diagram (log $|Z|$ vs log $f$) of titanium in various environment is shown in Fig. 3. Two plateau regions were observed on the curves in L929/MEM + FBS and MEM + FBS, while one plateau region was observed in MEM. These results indicated that proteins and cells formed a certain layer outside the surface oxide film of titanium with electric resistance. The impedance ($Z$) in the plateau region in lower frequency side in L929/MEM + FBS and MEM + FBS was slightly higher than that in MEM + FBS. That is, the presence of L929 affected the composition and structure of the L929/titanium interface. The biomolecule adsorption layer containing proteins and cells was formed on surface oxide film and

![Fig. 1. Titanium ion concentration in culture medium in which titanium disk and macrophage are incubated. Mφ: macrophage, HDP: high density polyethylene powder, AOE: antioxidant enzyme.](image1)

![Fig. 2. Schematic illustration of an electrochemical analysis instrument with culturing cell.](image2)

![Fig. 3. Bode diagram of titanium in various environments.](image3)
worked as a capacitor by preventing the diffusion of molecules. The cell enhanced this prevention.

3. Surface of metallic materials

Reactions between the surface of metallic materials and living tissues are the initial events when the materials are implanted into the human body. Tissue compatibility is governed by the reactions in the initial stage. In this regard, the surface properties of materials are important. The composition of the surface oxide film changes even though the film is macroscopically stable. Passive surfaces exist simultaneously in contact with electrolytes, undergoing a continuous process of partial dissolution and reprecipitation from the microscopic viewpoint [6]. Therefore, the surface composition should be changed according to the environment.

Immersion in simulated body fluids of metallic materials and surface analysis of the materials are the simplest technique to characterize the change in the composition and structure of the surface oxide film in the human body. Characterization of materials retrieved from animal and human tissues is in good agreement with the results.

Calcium phosphates are formed on titanium and its alloys by immersion in Hanks’ solution whose composition is similar to extracellular fluids and other solutions [7–10] as shown in Table 1. Hydrated phosphate ions are adsorbed by a hydrated titanium oxide surface during the release of protons [9]. Calcium ions are adsorbed by phosphate ions adsorbed on the titanium surface, and eventually calcium phosphate is formed. The above phenomena are characteristic in titanium and its alloys [7]. Also, phosphate ions are preferentially taken up during regeneration of surface oxide film on titanium, the film consists of titanium oxide and titanium oxyhydroxide containing titanium phosphate. Calcium and phosphate ions are adsorbed to the film after regeneration, and calcium phosphate or calcium titanium phosphate is formed on the outermost surface [11].

Five kinds of Co–Cr–Mo alloy specimens were prepared according to the following methods: polishing in de-ionized water, autoclaving, immersion in Hanks’ solution, immersion in a cell culture medium, and incubation with cultured cells. The surface oxide film of a Co–Cr–Mo alloy is characterized as oxides of cobalt and chromium with a small amount of molybdenum (Fig. 4) [12]. The Co–Cr–Mo alloy preferentially releases cobalt during a cell culture, and the resultant surface oxide film consists of chromium containing a small amount of molybdenum [12]. That is, the surface oxide film is reconstructed in living tissues.

4. Fatigue and fretting fatigue

Materials implanted in the human body are intermittently stressed with loads due to weight and action [2]. In particular, materials in the lower extremities are intermittently loaded with stress several times heavier than the body weight. In addition, loading is repeated in tremendous cycles. Such loads are applied in the chemical environment described earlier.

4.1. Fretting fatigue strength

The wear of metals used as medical devices usually occurs in the human body. Even in fixation devices such as plates and screws, micro-motions at the fixation site between plate and screw occur intermittently. This phenomenon is called fretting. Fretting is defined as ‘the relative oscillatory tangential movement of a small amplitude which may occur between contacting surfaces subjected to vibration or experiencing cyclic stressing, i.e. fatigue’. Causes of fractures in metallic biomaterials are mainly corrosion fatigue and fretting corrosion fatigue. The fatigue strength of stainless steel is influenced by partial oxygen pressure and/or biomolecules, but that of Ti–6Al–4V is not influenced [13], indicating an influence of corrosion. Therefore, fretting fatigue is also influenced with corrosion.

A test unit for fretting fatigue newly developed is shown in Fig. 5(A) [14–16]. A tensile fatigue test is carried out with attaching pads consisting of the same material so that contacting surfaces are reproduced. The temperature and dissolved oxygen concentration of the test solution were

| Materials          | Precipitates                          |
|--------------------|---------------------------------------|
| Ti, Ti–6Al–4V, Ti–56Ni | Calcium phosphate                      |
| Cr, SUS316L         | Chromium phosphate (later calcium phosphate) |
| Co–30Cr–5Ni, Ni–20Cr| Titanium phosphate                     |
| Ti–25Zr             | Zirconium phosphate (later calcium phosphate) |
| Ti–50Ni, Ti–60Ni    | Titanium phosphate                     |
| Ti–75Ni, Zr         | Zirconium phosphate                    |
| Au–9Cu–6Ag          | Chloride                               |
| Ag–20Pd–15Cu–12Au   | Only hydrated                          |
| Au                  |                                       |
controlled in this unit. Fig. 5(B) represents the stress–number of cycle ($S$–$N$) curves obtained with this unit in Ti–6Al–4V alloy (B).

PBS(−) is generally lower than in air because of the low oxygen partial pressure in PBS(−) except the Co–Cr–Mo alloy. Metal ions dissolved during a fretting fatigue test were quantified, and the results revealed that metallic materials may show toxicity with the release of ions and wear debris by fretting [17].

4.2. Dissolution of metal ions under fretting

When an alloy is abraded, metal ions are released. However, the kind of element and the amount released cannot be predicted from the nominal composition of the alloys. A large amount of molybdenum, which is a trace element in stainless steel, was detected in PBS(−) after the fretting fatigue test [17]. In addition, more nickel and manganese were detected compared to iron and chromium. In the fretting fatigue test of Ti–6Al–4V in PBS(−), titanium, which was a main component, was hardly detected [18]. Nickel, which is absent from chemical analysis data, and iron, which is a trace element, was detected from a filtrated solution. The chemical composition of Ti–6Al–4V and the concentration of dissolved metal ions in the PBS(−) are summarized in Fig. 7. These studies indicate that even trace elements in an alloy are not negligible from the viewpoint of metal ion release and safety.

Preferentially dissolved elements such as nickel are not used for the regeneration of a surface oxide film; elements used for the formation of the film, such as titanium, are hardly dissolved. The elements preferentially dissolved from an alloy have not been absolutely determined but have been somewhat determined among component elements of the alloy.

To investigate the mechanism behind the release of metal from titanium implants in vivo, bone–plate–screw sets consisting of pure titanium were implanted into the legs of rabbits for 48 weeks [19]. Four groups of experiments containing control were conducted: (1) the tibia cut artificially was fixed by one set of bone plate and screws, (2) the same set was implanted separately into muscles in the leg, (3) the set was fixed on the tibia and immediately retrieved, and (4) no implantation was performed. The amounts of titanium in all tissues from knee to ankle were...
quantified using atomic adsorption spectrometry. The ratio of amounts of titanium detected in the groups (1)–(3) was 100:10:43. No titanium was detected in the group (4). Causes of the release of titanium in the group (1) include that in the groups (2) and (3). Major causes of titanium release were surgical handling in implantation and wear and/or fretting during experimental-term for 48 weeks. Titanium was also released in the absence of wear.

5. Cytotoxicity of metals

Metallic materials implanted in the human body rarely induce serious conditions. Metallic materials conventionally used in medicine and dentistry does not show toxicity. However, some component elements of alloys show toxicity. The toxicity of a metallic material is governed not only by its content and the toxicity of component elements of the material but also by its corrosion and wear resistance. Toxic elements must be excluded from alloying elements in the development of new alloys.

The cytotoxicity of 43 metal salts is systematically evaluated by colony formation method using two kinds of cultured cells, L929 fibroblasts and MC3T3-E1 osteogenic cells. The IC50 values (50% of cell growth inhibition), which ranged from 1.36 × 10⁻⁶ to 1.42 × 10⁻² mol/l, were calculated [20]. The rank orders of cytotoxicity in L929 are summarized in Table 2. In addition, the cytotoxicity of 12 metal salts was evaluated using four cell lines, and the results were compared with the above study. The correlation was statistically proved by the IC50s of 12 metal salts among these cell lines, suggesting the existence of a generic tendency to metal salt cytotoxicity beyond cell lines [21].

Mutagenicity is a very fundamental and important toxicity related to carcinogenicity and reproductive/developmental toxicity because the damages to genes or DNA can be a cause of carcinogenesis and developmental abnormalities. Forty-one metal salts encompassing 36 metals and five metallic elements tested with different valences, were evaluated on their mutagenicity using a microbial test, the umu test, to obtain the systematic data necessary for metal ion mutagenicity. As a result, K2Cr2O7, RhCl3, IrCl4, and MgCl2 are positive without S-9 mix, which is a mixture of the micro-some fraction of a rat liver with coenzymes such as reduced nicotinamide adenine dinucleotide phosphate and reduced nicotinamide adenine dinucleotide [22]. K2Cr2O7, RhCl3, IrCl4, CuCl, CuCl2, and VCl3 are positive with S-9 mix.

According to the quantification of metallic element in PBS(−) after fretting fatigue test and cytotoxicity test of the solution, the possibilities that metallic biomaterials such as 316L stainless steel, titanium, Ti−6Al−4V alloy release metallic ions or debris when they are implanted into the place inside a body where fretting fatigue occurs and that metallic elements in 100 ppb released from the materials have toxic effects for cells are pointed out [17,18].

6. Biocompatibility: cell adhesion to materials

Affinity for cells is one of the important properties for biomaterials, because they are always used adjacent to living tissue. For the development of new biomaterials with superior biocompatibility, quantitative evaluation of the materials’ affinity for cells is necessary. One method to evaluate the materials’ affinity for cells quantitatively is to measure the detachment force of an adherent cell on a material.

A new system was developed to measure directly the shear force necessary to detach a cell from a material [23]. The detachment force was measured in the cell culture medium by applying a lateral load to the cell which adhered to the material using a microcantilever. The cell was observed using an optical microscope and the image of the cell was recorded on a video tape through a CCD camera during the measurement. The cell adhesive area before detaching the cell was measured by analyzing the obtained video image of the cell. The schematic illustration of the system is shown in Fig. 8.

The shear force and the total energy to detach L929 fibroblast from glass, ploystyrene, and fibronectin- or

Table 2

The rank orders of cytotoxicity of metal ions in L929 and MC3T3-E1

| L929 | Cr⁶⁺ > Cd⁴⁺ > V⁵⁺ > Ag⁺ > Hg²⁺ > Hg⁰ > Ti³⁺ > Ga³⁺ > Cu²⁺ > Mn²⁺ > Co²⁺ > Zn²⁺ > Be²⁺ > Ni²⁺ > Sn⁴⁺ > In³⁺ > Ir³⁺ > Ti³⁺ > Pd²⁺ > Y³⁺ > Cu⁺ > Rh³⁺ > Pb²⁺ > W⁶⁺ > Cu¹⁺ > Br⁻ > Ti³⁺ > Cs⁺ > Hf⁴⁺ > Mo⁴⁺ > Zr⁴⁺ > Ta⁵⁺ > Ba²⁺ > Rh⁴⁺ > Nb⁵⁺ > Al³⁺ > Fe³⁺ > Ru³⁺ > Fe³⁺ > Sr⁴⁺ > Li⁺ > Sn⁴⁺ > MC3T3-E1 | Cd³⁺ > In³⁺ > V⁵⁺ > Be²⁺ > Sn⁴⁺ > Ag⁺ > Hg²⁺ > Cr⁶⁺ > Co²⁺ > Bi⁴⁺ > Ir³⁺ > Cu¹⁺ > Hg⁺ > Cu²⁺ > Rh³⁺ > Ti³⁺ > Sn⁴⁺ > Ga³⁺ > Pd²⁺ > Cu⁵⁺ > Ni²⁺ > Zn²⁺ > Y³⁺ > W⁶⁺ > Fe³⁺ > Pd²⁺ > Fe³⁺ > Ti³⁺ > Hf⁴⁺ > Ru³⁺ > Sr⁴⁺ > Sn⁴⁺ > Ba²⁺ > Cs⁺ > Nb⁵⁺ > Ta⁵⁺ > Zr⁴⁺ > Al³⁺ > Mo⁴⁺ > Rb⁺ > Li⁺ |

Fig. 8. Principle of the measurement of a shear force for cell detachment from a material using a newly developed instrument and force displacement curves obtained with the instrument.
collagen-coated polystyrene were measured directly using the new system [24]. Cell adhesive shear force, cell adhesive shear strength, cell detachment energy, and cell detachment surface energy are the highest in this order: the cells on collagen-coated polystyrene, those on fibronectin-coated polystyrene, and polystyrene and glass, that is good agreement with empirical data. Cell adhesive shear strength and cell detachment surface energy depend on the number of the bindings between the cell and a material’s surface rather than on the strength of each bindings.

Adhesive properties of L929 to sputter-deposited metal films depend on the kind of metals to which they adhere as shown in Fig. 9. Among titanium, chromium, aluminum, gold, silver, and palladium, the cells on chromium and titanium had the highest cell adhesive shear strength and the cell detachment surface energy which were almost the same to those to glasses [25]. The cells on other metals which are dissolved in a cell culture medium had lower cell adhesive shear strength and the cell detachment surface than the cells on glass. A metal which has high corrosion resistance in medium, low cytotoxicity caused by released metal ions, a stable surface oxide layer, and a moderate hydrophilic surface will have affinity for cells.

7. Future of metallic biomaterials

Metallic materials are widely used in medicine not only for orthopedic implants but also for cardiovascular devices and other purposes. Biomaterials are always used in contacting tissues. Therefore, inter-reactions among material surfaces and tissues must be well understood; this knowledge is essential to develop new novel materials. All evaluation techniques must be performed with this concept. In particular, reactions between metal surface and biomolecule and that between metal surface and cell reactions are important.

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