Corrosion of Metallic Biomaterials in Cell Culture Environments

by Sachiko Hiromoto

Metallic materials are used in biomedical devices for various parts of the human body (Fig. 1). The implanted material is exposed to body fluids, such as intercellular fluid and blood depending on the surrounding tissue. Body fluid consists of inorganic ions (Na^+, Cl^-, Ca^{2+}, $H_xPO_4^{n-}$ *etc.*), amino acids, proteins, and organic acids. The pH of body fluid is buffered at 7.15~7.35 but it decreases down to 5.2 during an inflammation reaction.[1] The concentration of oxygen depends on the part of body: in the intercellular fluid and arterial blood, it is 1/80~1/4 and 2/3^{rds} of that under ambient conditions, respectively.[2] The kind of cell in the surrounding tissue depends on the part of body and varies as time proceeds after implantation. Just after implantation of the material, macrophages assemble around the implanted material as the initial stage in a foreign body reaction. Because macrophages secrete active oxygen species, which chemically attack the surface oxide film of metallic materials, the influence of the presence of macrophages has been investigated.[3,4]

Mu, *et al*. reported the importance of the coexistence of foreign bodies based on the result that the acceleration of dissolution of pure Ti was enhanced by the coexistence of polyethylene wear debris.[3] This is because the stimulation by a foreign body activates macrophages to secrete active oxygen species. Lin, *et al*. reported that the presence of macrophages alone does not always accelerate the corrosion of metallic biomaterials.[4] Besides active oxygen species, macrophages secrete fibroblast growth factor, inducing fibroblasts, which produce collagen and polysaccharide for encapsulation of the implanted material. The thickness of capsular tissue depends on the biocompatibility and the size of material. With the cure of inflammation, the macrophage disappears within a few weeks and the cells originating from the surrounding tissue adhere to the surface of material. The cells use cell adhesive proteins to adhere to the surface of the material and produce an extracellular matrix (ECM) with a characteristic composition depending on the kind of cell (Fig. 2). For example, osteoblasts produce ECM, encouraging the formation of new bone consisting mainly of collagen and hydroxyapatite (HAp). In the end, the implanted material is surrounded by tissue depending on the part of body. In the case of artificial joints, bone fixation devices, and dental implants, the surface of the material attaches to both hard and soft tissues. Orthodontic wires, crowns, and bridges are used in oral cavities whose environments vary drastically. Stents, clips, and artificial hearts for circulatory diseases are exposed to blood and soft tissue.

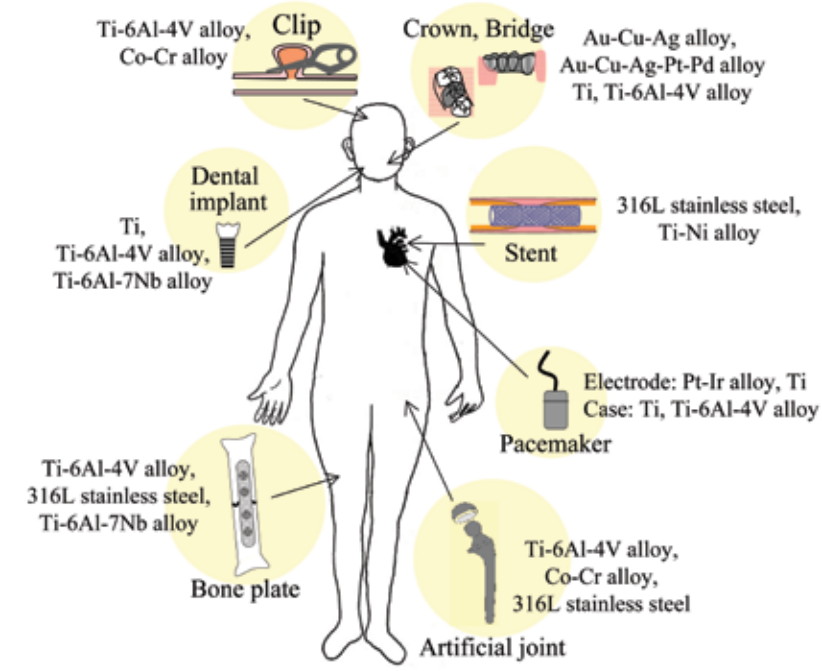

Fig. 1. *Metallic devices and metallic biomaterials used for them.*

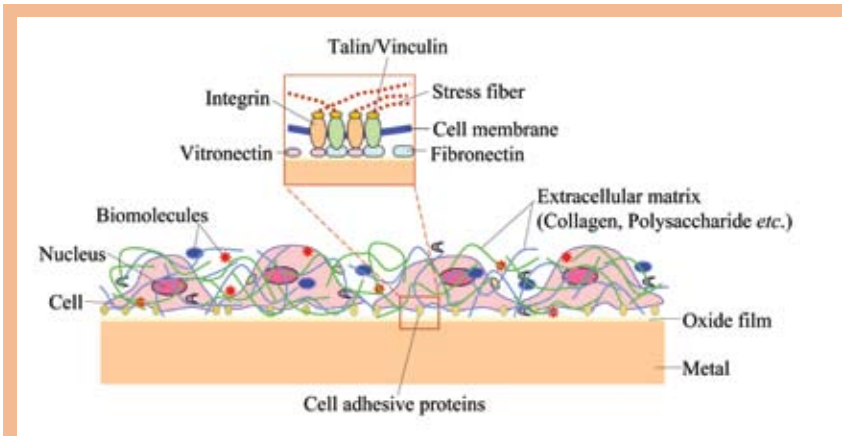

Fig. 2. *Illustration of cells adhering to the surface of a metallic biomaterial.*

Failure of artificial joints and bone fixation devices such as fracture and loosening becomes notable after 5-10 years of implantation.[5] These mechanical degradations are often caused by fatigue, fretting fatigue, and wear accelerated by corrosion. This fact suggests that the corrosion progresses little by little for many years on the surface of material attaching to the surrounding tissue. Thus, the corrosion factors in the tissue, which will attach to the implanted material, should be elucidated to improve and evaluate the corrosion resistance of metallic biomaterials.

Dental implants and coronary stents were developed in the last few decades, and Zr alloys and Ta were relatively recently applied to the acetabular head and stem of artificial joints, respectively, in addition to the most commonly used metallic biomaterials such as Ti-6Al-4V alloys, 316L stainless steel, and Co-Cr alloys. Moreover, various types of surface modifications were developed to improve corrosion resistance and biocompatibility and to add drug eluting properties. For surface modification, ceramics (HAp, bioglass, *etc.*), polymers (polyethylene glycol, 2-methacryloyloxyethyl phosphorylcholine polymer, *etc.*), and several biomolecules are used. On the other hand, the proper evaluation environment and techniques, taking into consideration the characteristic factors in the environment to which the materials will be exposed, have not yet been developed for these relatively new usages of metallic biomaterials.

Regardless of the kind of devices and the part of the body, the corrosion resistance of metallic biomaterials is evaluated in saline, phosphate buffer saline solution, simulated body fluid containing only inorganic ions, and medium with and without the addition of serum. The function of modified surfaces such as drug eluting properties and bone conductivity is evaluated in the same solutions. Thus it is not surprising that *in vitro* test results do not always correlate with processes occurring in the human body.[6] This problem may be due to the fact that the above test solutions were not formulated based on elucidation of the interaction between tissues and the surface of metallic materials. As mentioned above, a certain kind of cell adheres to the surface of implanted materials to elicit characteristic molecules and chemicals that form tissue around the material. The presence of cells changes the environment on the material's surface. For example, the variety of adsorbed organic molecules on pure Ti and 316L steel in the presence of a fibroblast was different from that seen when the material was immersed only in medium.[7] This leads to many questions: Is the evaluation using the above-mentioned simple solutions sufficient for various metallic biomaterials that will be used in various parts of the body? Do the molecules and chemicals produced by the cells accelerate the corrosion of metallic biomaterials? Does the existence of cell bodies and ECM influence the diffusion of ions and molecules since the ECM can form a gel? Does the surface, which accelerates the chemical precipitation of HAp, also encourage bone formation by bone cells?

Because we do not have a link connecting the results of the simple solutions and those obtained *in vivo*, animal tests are carried out before applying new materials for practical use, to secure data on material reliability. However, the individual differences in the animals and operator variability inevitably skew the results. These errors prevent the elucidation of the interaction between the tissue and the material's surface. Importantly, the number of animal test must be limited. Elucidation of the interaction reaction is necessary to develop *in vitro* evaluation environments and techniques that can sufficiently simulate the *in vivo* environment. We supposed that culturing cells on the surface of the material could simulate the *in vivo* environment in some way.

Surface Composition of Metallic Biomaterials

To examine the environment formed on the surface of metallic biomaterials in cell cultures, the surface composition of Ti-6Al-4V alloy and the 316L steel of bone plates and intramedual rods retrieved from patients was compared to that of pure Ti and 316L steel immersed in medium (Eagle's minimum essential medium with the addition of 10 vol% fetal bovine serum) with and without fibroblast L929 for 7d. No significant corrosion was observed on the retrieved materials. Both sides of the retrieved implants attached to hard and soft tissues were analyzed using X-ray photoelectron spectroscopy.

Calcium phosphate and sulfide/sulfite, in addition to oxygen and alloying elements, were detected on the surface of Ti and 316L steel immersed in medium with fibroblasts. The presence of fibroblasts caused the decrease of precipitated calcium phosphate and altered the chemical state of sulfur. Very similar elements were detected on the Ti-6Al-4V alloy and 316L steel implants retrieved from patients.[8,9] While the variety of elements precipitated in soft tissue was the same as that in hard tissue, the amount of calcium phosphate precipitated in soft tissue appeared to be less than that in hard tissue. The sulfide/sulfite observed was supposed to originate from adsorbed biomolecules produced by cells because the chemical state of sulfur precipitated in the medium without fibroblasts was sulfate, but that with fibroblasts was sulfide/sulfite.

It has been reported that cultured osteoblasts form bone-like tissue on the surface of HAp have a very similar structure to that formed in animal bodies.[10] These facts indicate that the *in vivo* environment on the surface of the material can be simulated at a certain level by culturing cells on the surface of material.

Electrochemical Measurements in Cell Culture Environments

For electrochemical measurements in cell culture environments, an electrochemical cell was developed.[11,12] To avoid contamination by bacteria, all the equipment, electrodes, and specimens must be sterilized. The contaminated bacteria produce detrimental substances, which may influence the corrosion of metallic materials, not just kill the cultured cells. The easiest technique for sterilization of specimens is autoclaving although it causes the growth of surface oxide films on metallic materials.[13] For practical use, ethylene oxide gas is commonly used; however, this toxic gas requires careful handling. UV irradiation is also employed, but the UV light does not reach the inner part of the polishing scar and pore on the surface. To allow cells to adhere to the surface of the specimen, the specimen should be fixed horizontally to the electrochemical cell. Then the cells should be seeded homogeneously because the cell density influences the behavior of the cells. Observation of cultured cells is desirable to check the biocompatibility of the material as well as the cell coverage. However, the living transparent cells on the metallic material cannot be observed by optical microscope. *In situ* observations using fluorescent labeling of a certain protein in the cell body or observation after the experiment with fixation and staining are recommended.

Potentiostatic polarization measurements were carried out on pure Ti after culturing a different number of fibroblast L929 in the medium for 7d (Fig. 3).[12] On the cathodic polarization curve, the current density in the potential region between -0.2 and -0.5 V (SCE) decreased with an increase in the number of cells. The decrease of cathodic current density in the same potential region with the presence of fibroblasts was also observed on 316L steel.[14-16] In this potential region, the cathodic reaction corresponds to the reduction of dissolved oxygen. Gerbert, *et al.* reported that the diffusion of dissolved oxygen was retarded near osteoblasts cultured on Ti.[17] Therefore the following causes were supposed for the decrease in the reduction of dissolved oxygen. While dissolved oxygen near cells decreased with the consumption of oxygen in the metabolism of cells, the reach of dissolved oxygen to the material surface was retarded by cell bodies and ECM. As a result, the concentration of dissolved oxygen is kept rather low on the surface of material.

No significant difference in passive current density was observed with a different number of fibroblasts on Ti. The passive current density of Ti and Co-Cr alloys was not influenced

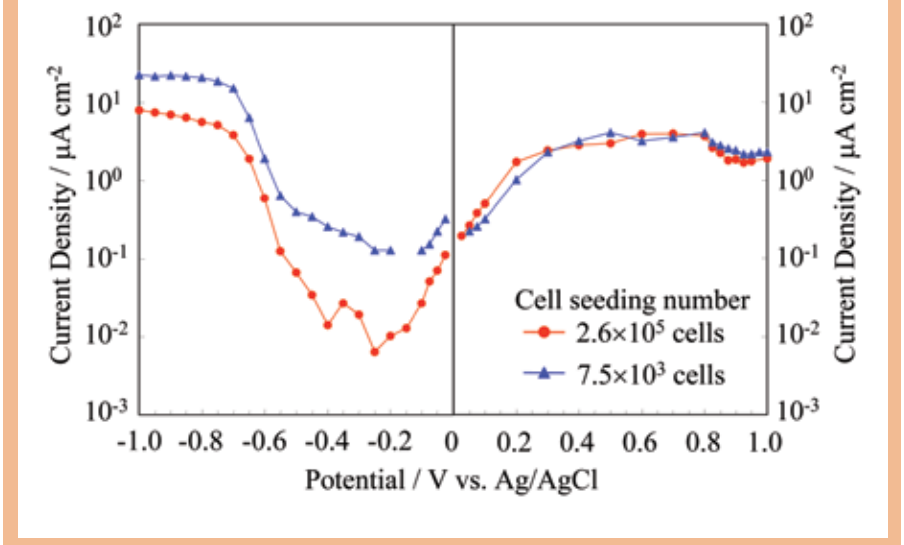

Fig. 3. *Polarization curves for pure Ti in the presence of a different number of fibroblast L929 after culturing for 7d.*

by the presence of fibroblasts.[8] In the case of 316L steel, the presence of fibroblasts caused the decrease of the protectiveness of passive films, which was shown by an increase of passive current density and a decrease of pitting potential.[15] To understand the decrease of protectiveness of passive films, the pH of the medium near the fibroblasts cultured on 316L steel was measured. The pH near the cells was found to be lower than that of the bulk medium.[18] This is attributed to acidification by the accumulated dissolved metal ions (cations) near cells under the diffusion limited environment indicated by cathodic polarization behavior. This fact indicates that the pH of medium near cells on material was decreased by the acidification, leading to the initiation of crevice corrosion. The accumulation of dissolved metal ions around the implanted metallic materials was reported by Uo, *et al.*[19] indicating that the pH of the body fluid around the implanted material is kept lower than the constant pH around neutral.

Impedance measurements were carried out on pure Ti and 316L steel with and without culturing fibroblasts L929 for 7d. The retardation of diffusion near cells on the material was suggested by the results of the polarization measurements. An equivalent circuit model having a Warburg impedance element was assumed. The Warburg impedance obtained by data fitting showed a significant increase in the presence of fibroblasts.[14,15] Both the results of the polarization and impedance measurements indicate that the presence of a fibroblast retards the diffusion of ions and molecules, leading to a change in the chemical environment on the surface of metallic biomaterials (Fig. 4): the concentration of dissolved oxygen and the pH of the medium near cells decreases. Simultaneously, the accumulated dissolved metal ions may induce chloride ions near cells like the initial stage of crevice corrosion.

The environment formed by fibroblasts is expected to be different from that by osteoblasts or endothelial cells. Thus the different kinds of cells may show different influences in corrosion. To develop a proper and simple evaluation environment and technique for metallic biomaterials, depending on the part of the body, further investigation using various kinds of cells is required. At last, the following are noted for using established cell lines. The established cells are the peculiar cells which proliferate without limitation and whose characteristic does not change through the passage of many generations. It means that the established cells lose their original function and characteristic so that they cannot completely reproduce the *in vivo* environment. Additionally, the cultured cells on the material surface form a 2D layer, whereas the tissue in the body has a 3D structure.

Corrosion of Bioabsorbable Metallic Materials

The application of magnesium alloys such as WE43 and AE21 to bioabsorbable devices was attempted.[20] The bioabsorbable device is required to degrade and disappear immediately after the affected part is cured enough to bear the load by itself because the remaining device causes the restenosis of blood vessels or bone resorption. Thus, the control of *in vivo* degradation speed of device will determine the success of the application. Degradation, *i.e.*, corrosion, of magnesium alloys is expected to be considerably influenced by the diffusivity on the surface of the alloy, because the corrosion of magnesium alloys leads to the increase of pH, while the solubility of magnesium oxide and hydroxide depends on pH sensitivity. Lévesque, *et al.* suggested the importance of the existence of blood flow to evaluate the degradation of magnesium alloys for coronary stent use.[21] It has been reported that the corrosion rate of pure magnesium in a sodium chloride solution is so rapid that the initial growth of the surface hydroxide/oxide film under static conditions seemed to be more rapid than that under dynamic conditions.[22] In animal testing, a magnesium alloy stent is covered with endothelial cells,[20] which will also influence the degradation speed of the magnesium alloy. To control the degradation speed, various surface treatments will be developed in the near future. Therefore, the establishment of a proper evaluation environment and techniques for bioabsorbable magnesium alloy is required.

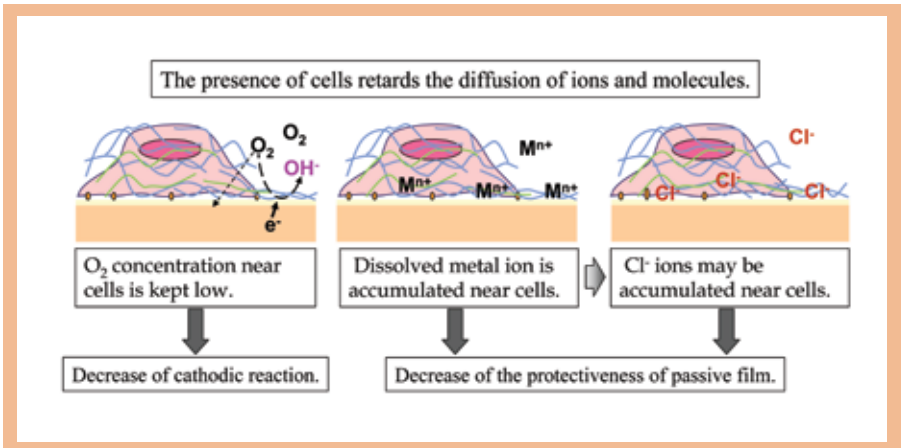

Fig. 4. *Influences of the presence of fibroblasts on the corrosion of metallic biomaterials.*

Summary

The need for reliability and functionality in metallic biomaterials is increasing. On the other hand, evaluation of corrosion behavior and other surface properties is currently carried out in simple physiological solutions formulated without examining the influence of characteristic factors *in vivo*, especially in the presence of cells. The resulting problem is that the corrosion behavior obtained in these simple solutions does not always correlate with that observed *in vivo*.

Electrochemical measurements of metallic biomaterials in a cell culture environment using fibroblasts revealed that the presence of fibroblasts retards the diffusion of ions and molecules near the cells on the surface of materials. The diffusion retardation leads to the decrease of cathodic reaction of dissolved oxygen on several metallic biomaterials. Further, it sometimes leads to a decrease in the protectiveness of the passive films of metallic biomaterials like 316L steel having a rather lower corrosion resistance than Ti. This is because the accumulation of dissolved metal ions can cause medium acidification. These results show that the presence of cells is not a negligible corrosion factor.

Therefore, to accurately evaluate and to encourage the improvement of surface properties, the (surface) interaction between metallic biomaterials and the tissue to which the material will attach, should be elucidated. Understanding the characteristic corrosion factors in each part of the body is necessary to develop a simple but sufficient environment for the evaluation of various surface properties. Electrochemical measurements in a cell culture environment are useful for such an elucidation.[24,25]

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