The Application of the Self-Assembled Monolayers for Improving the Bioactivity of Biomaterials

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THE APPLICATION OF THE SELF-ASSEMBLED MONOLAYERS FOR IMPROVING THE BIOACTIVITY OF BIOMATERIALS

CHAO LIU
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Chapter 1

GENERAL INTRODUCTION

1. Present Status of Biomaterials

Biomaterials, generally defined as materials used in medical applications, have been used since ancient times. There are various advances in the biomaterials field during the last 50 years. The most important requirement for the choice of the biomaterial is its acceptability by the human body. In order to have long-term usage in the body without rejection, a biomaterial used for implants should possess some important properties. Hench [1] classified different generations of biomaterials based on their properties, which are known as first generation (bioinert materials), second generation (bioactive and biodegradable materials) and third generation (materials to stimulate specific responses at the molecular level). And the most common classes of materials used as biomedical materials are Metals, Polymers, Ceramics, and Composites as shown in Table 1-1 [1-5].

With the significant increase in application areas, these materials are used either as such or in combination to form most of the implantation devices available today. At the same time, these days’ biomaterials are information rich along with biologically active components derived from nature. In the near future, biomaterials will surely have an even greater role in medicine in form of implants (sutures, bone plates, joint replacements, etc.) and medical devices (pacemakers, artificial hearts, blood tubes, etc.), thus will improve the quality of life of the patients.

1.1 Metal as biomaterials
Stainless steel (SS) and cobalt–chrome (Co-Cr) based alloys were the initially used metallic materials during the twentieth century in orthopedic applications successfully. Titanium (Ti) and its alloys were introduced in the 1940s. Later in the 1960s the shape memory alloys such as nickel-titanium (NiTi) appeared and they opened a whole new range of applications because of their special mechanical behaviour. Due to their high elastic modulus, they are widely used as the metallic devices which could take most of the load when contacting with bone. At the same time, they can produce stress shielding in the adjacent bone which induces its resorption that will lead to the eventual failure and loosening of the implant [6, 7].

SS materials show resistant to a wide range of corrosive agents because of their high chromium (Cr) content (more than 11 wt.%) which results in the formation of a corrosion resistant coating of oxide Cr$_2$O$_3$ [8]. Some important discoveries such as cemented prosthesis with a stem made of stainless steel was the first total hip prosthesis designed by Charnley in the very late 1950s [2]. Also, biomaterials based on SS got popular in traumatological temporary devices such as fracture plates, screws and hip nails, owing to their relative low cost, availability and easy processing.

Co-Cr based alloys have superior mechanical properties such as they have a high elastic modulus (220–230 GPa) similar to that of stainless steel (approx. 200 GPa). Besides, they have excellent corrosion resistance and most significantly their fatigue strength. These properties led to the introduction of Co–Cr–Mo alloy (ASTM F75, Vitallium) in hip prostheses [3, 4]. Also, they have been used in combination with polyethylene (PE) for artificial disc prostheses such as the SB Charite artificial disc replacement system [5].

Similarly, Ti and its alloys are attractive in the biomedical field, due to their excellent properties such as high corrosion resistance, high biological compatibility and a low density (approx. 4700 kg/m$^3$). Ti and its alloys show excellent corrosion behavior due to the formation of an adhesive TiO$_2$ oxide layer on their surface. The dental and surgical applications of Ti alloys
was started by Branemark [9] named as the osseointegration phenomenon for Ti implants. Although Ti and its alloys combining a range of excellent properties, their processing is not easy whether it is machining, forging or heat treating.

Other than conventional metallic materials, the shape memory effect was discovered in NiTi alloys by Buehler & Wang (1967) [10] in the 1960s. It is the ability of a material to recover its shape upon heating after having been ‘plastically’ deformed. Their use in load-bearing applications is comparatively better than other metallic materials [11]. Ability to deliver a uniform compressive stress and to produce constant force over a large displacement could make them useful in fracture repair [12], anchoring of prostheses [11] and for bone distraction devices. But the problem associated with their application is allergy and toxicity with the release of Ni ions, which has hampered their use.

1.2 Ceramic biomaterials

Ceramic biomaterials (bioceramics) are the ceramics used for repairing and replacing the diseased or damaged parts of the musculoskeletal system. They are also playing an important role among all the biomaterials. For example, bioglasses (BGs), glass–ceramics and calcium phosphates (CaPs) both as ceramics and cements are commonly employed ceramic materials. The application of these materials as bone substitutes started around the 1970s [13-16] and have been mainly used as bone defect fillers [17]. Alumina, zirconia and several porous ceramics are also the most commonly used ceramic biomaterials. These are inorganic materials with limited range of compositions. The parameters such as sintering temperature, purity, size and shape of the powder precursor significantly influences the microstructure and properties of the produced ceramics. Bioceramics demonstrated their applications in replacement of the traditional metallic femoral heads of hip prostheses by high-density and highly pure alumina (\(\alpha\)-Al\(_2\)O\(_3\); [18]). Also, they are used for the acetabular cups due to low wear rates, excellent corrosion resistance, good
biological compatibility and high strength [19]. However, ceramic material components showed early failures due to their low fracture toughness. Therefore, their design and production processes have been improved for better material property.

2. Bioactive materials

Bioactive materials are the materials which can spontaneously bond to the living bone tissues. Examples are bioactive glasses, bioactive ceramics, bioactive glass-ceramics, bioactive calcium phosphate ceramics and some bioactive coatings and composites [20]. Figure 1-1 shows the example of bioactive materials having a direct bonding with the bone via apatite layer [21].

When mentioned about bioactive materials, it firstly comes to the bioactive ceramics, which have been claimed to be highly osteoconductive. HA (Ca_{10}(PO_4)_6(OH)_2), beta-tricalcium phosphate (β-TCP, Ca_3(PO_4)_2), 45S5 Bioglass-type glasses and glass-ceramics A-W are commonly used bioactive ceramics. On the basis of their different synthesis process, these materials show different physical and chemical properties [22]. HA exhibits good bioactive properties but its chemical stability reduces solubility rate when compared to TCPs. Therefore, HA may remain integrated into the regenerated bone tissue after implantation, whereas TCPs are completely reabsorbed [23, 24]. Some material such as silicon and TiO_2 also plays an essential role in bone formation [25, 26]. Silicon ions are known to have effect for enhancement of calcification of young bone [27]. Thus, the addition and release of silicon into various ceramic biomaterials have been extensively investigated. In fact, it has been proposed that the introduction of silicon into apatite increase the ability to induce bone tissue than non-doped apatites [28]. It is due to the formation of Si-OH on the surface which enhances the bioactivity and thus initiates the nucleation, therefore improves the material-bone bonding. There are reports on SiO_2 and TiO_2 gel inducing nucleation of carbonated HA (bone like layer) on the biomaterial surface. In several cases, TiO_2 has also been used as a component of various glasses.
to enhance chemical durability [29, 30]. Apart from their applications mentioned above, there are some drawbacks also associated with them owing to their poor mechanical properties, low tensile strength and very low fracture toughness, which makes them not suitable for loadbearing applications. For this BGs are better materials and popularly used in low load-bearing material applications such as for one repair in dental and orthopaedic surgery and for middle ear bone [31-33].

Ti and its alloys have been also utilized for the development of porous metallic scaffolds for bone tissue engineering and drug delivery systems [34]. Similarly, tantalum metal because of its excellent in vivo biocompatibility, porous tantalum with its high volumetric porosity, low elastic and good frictional characteristics is also being used clinically in several orthopaedic applications and an ideal candidate for weight-bearing applications such as total joint arthroplasty [35]. Titanium, its alloys and tantalum can become bioactive via a simple chemical treatment and increasingly used in the medical applications [36].

2.1 Why bioactivity is important?

The appropriate selection of a material for a specific application is generally achieved by matching the material properties with the required ones. However, in the case of biomaterials, biological requirements needed to be include. Therefore, various factors such as foreign body reaction, stress shielding, biocompatibility and bioactivity should be taken into account for design of implant devices. In this category, the materials first were supposed to have a suitable combination of physical properties to match those of the replaced tissue with minimum toxicity. Later there was the development of their ability to interact with the biological environment and also the tissue/surface bonding. Basically, bioactivity is the interaction or effect that materials exert on cells for activating them to specific responses and behaviours. The bioactivity of the
biomaterial can achieve a natural bonding junction between it and the bone tissues by interfacial bonding layers [37].

2.2 Approaches involved to induce bioactivity

For introduction of good bioactivity, there must be similarities between the bone mineral phase. Also, their structural and surface features are responsible for good bioactive properties which enable binding to the bone with no fibrous connective tissue interface [38, 39].

The in vivo deposition of hydroxyl apatite (HA) at the material surface is an effective way to introduce the bioactivity or improve it. During the period of mid 1980s, these bioactive materials which include several bioactive glasses (BGs), ceramics, glass–ceramics and composites were in clinical use for variety of orthopaedic and dental applications.

Metallic materials used in orthopaedics are bioinert by nature. But approaches such as coating the surface of the implant with a bioactive ceramic (HA and BGs) and chemically modifying the surface of the material can be implemented to obtain bioactive metals. Some of the coating methods such as electrophoretic deposition [40], plasma spraying [41, 42] radio frequency or ionic ray sputtering [43], laser ablation [44, 45] or hot isostatic pressure [46] falls under the category of first approach methods. These methods however cannot produce covalent links with the substrate, and also the majority of them are not cost-effective. HA coating by plasma spray method is the most popular for biomedical applications. In the second approach chemical modifications have been developed to obtain apatite or other calcium phosphate (CaP) material layers on metallic surfaces which creates a direct chemical link between the substrate and the coating. Such methods have been developed for Ti and its alloys. It involves a thermochemical treatment which starts with the etching of surface material with an aqueous solution of NaOH, followed by a heat treatment at 600°C. A thin titanate layer thus formed able to create a dense bone-like apatite layer when placed in a simulated body fluid (SBF) [47].
Another method which involves chemical etching with hydrogen peroxide containing small amounts of tin chloride at 600°C [48] also been reported. Some other techniques such as the dipping the material in a sol–gel solution previously prepared at ambient temperature, followed by a thermal treatment at 500°C [49] can also be implemented. Also, the use of self-assembled monolayers (SAMs) designed with a functional group at their end is quite popular way to induce the nucleation of a CaP [50-52]. Some other method involves precalcification of the metallic surfaces by immersion in solutions of Na₂HPO₄ and Ca(OH)₂, then a two-step chemical treatment using mixed solution of HCl and H₂SO₄ followed by immersion in boiling NaOH solution [53]. Among all the surface treatments the main parameters are electrostatic charges and zeta potential, density of hydroxyl groups and structure of TiO₂ oxide [35, 36, 54, 55] which are mainly responsible for apatite formation.

Further these above mentioned methods have been modified time to time to affect the cells adhesion and subsequent proliferation and differentiation rates and to construct tight long-term integration between materials and tissue [56, 57]. Some examples are such as the formation of covalent chemical binding of biomaterials and biomolecules was formed through silane-treated titania surfaces, using amino- and carboxyl directed immobilization mainly through glutaraldehyde chemistry, and photochemistry by graft polymerization of biomolecules with a photoactive group [58, 59].

3. Self-assembled monolayers (SAMs) for biomaterials

The first fabrication and characterization of monolayer assemblies were performed by Nuzzo and Allara in 1980s [60, 61], for oriented organic molecules dramatically changed the surface science. In supramolecular chemistry SAMs, structures are ordered organic films [62] connected through non-covalent interactions such as various chemical and physical bonding, and metal co-ordination. SAMs are formed by the chemical adsorption of an active organic coating
on a solid surface. Various metal surfaces (e.g., Au, Cu, Ag, Pd, Pt, Hg and C) and surfaces with function of semi-conductor (e.g., Si, GaAs, indium coated tin oxide etc.) have been functionalized by organic molecules. A SAM is composed of layer of molecules with a “head group” at the end with affinity for a substrate. The other end is a terminal functional group combined with a “tail” as shown in Figure 1-2 [63]. The end functional groups (tail groups) could be modulated, and generally properly chosen to improve hydrophilic and hydrophobic properties of the substrate. The chain length, the adsorbate and the substrate affects the final organization of SAM layer. Packing density of the film is mainly decided by the steric hindrance and metal substrate properties whereas the chain length controls the SAM thickness [64]. In addition, external factors such as surface roughness and defects cleanliness of the substrate, method of preparation, and purity of the adsorbates also affects the SAM layer properties. However, sometimes due to the absorption of various kinds of unwanted molecules makes SAM highly unstable.

Various biomolecules such as proteins, peptides, DNA, carbohydrates, antibodies and therapeutics have been combined with SAMs for biomedical applications [65]. Therefore SAMs are highly attractive for modifying and newly exhibiting surface functions on various material surfaces.

Most of metals and their alloys with high reactivity are already covered with passive oxide layer with 10 nm in thickness and it has potential to protect the bulk metals [66]. The oxide layer provides a highly biocompatible surface and achieves integration with tissue suitable for dental implants or protheses. Some reports of SAMs on Ti [67], stainless steel [68], tantalum [69], zirconium [70] or nitinol [71] have been studied. Generally for metallic surface modification, the attaching group of phosphonic acids or silanes is used. However, the use of chemical and physical stability of SAMs is strongly governed by several factors such as reagents
for SAMs formation, morphology and chemical composition of substrates and kind of functional groups of the SAMs.

Typical process of SAMs formation is gas phase production and liquid phase production [71]. Growth from the gas phase needs equipment [72]. In the traditional preparation of SAMs growth via liquid phase production is simple dippings of cleaned substrate into the corresponding diluted solution [73].

4. Motivation for current research

In the last 60 years, biomaterials for orthopaedic applications have developed to biologically and mechanically match properties of body tissues and exhibit long lifetime in the body. During this, modification and control of surface properties at the micro/nano level has been quite important key technique. Cobalt-chromium (Co-Cr) alloy and alumina (Al₂O₃) both are bioinert materials i.e. they cannot induce apatite in their native state. However, the properties such as high strength, ductility, malleability, and temperature endurance, pitting resistance, crevice corrosion resistance and wear resistance make Co-Cr alloy an important biomaterial. Similarly, alumina shows excellent wear rates, excellent corrosion resistance, good biocompatibility and high strength. Therefore the SAMs films may be an important innovative and alternative technique to the traditional surface treatments able to improve the bioactivity.

In the current report my work will be mainly focused on the use of SAM monolayers for inducing the bioactivity of the metallic Co-Cr alloy and alumina (Al₂O₃). I will discuss mainly the following points.

In the first part of the work, I will introduce Co–Cr alloy as an efficient biomaterial. Co-Cr alloy has excellent wear resistance and is stable due to the formation of a passive, tenacious, self-replenishing chromium oxide, a few atomic layers thick. Cr–Co alloys have been attracting much attention for metal bearing surfaces of hip joints due to good wear resistance and high
hardness. However, Co-Cr alloy is a poor biomaterial, hence in this part I focused on making it a suitable bioactive material.

Firstly, my work started from inducing apatite layer formation process by using 10-carboxydecylphosphonic acid (10-CDPA) as SAM layer. 10-CDPA is one of the phosphonic acids, which are somewhat less often characterized compared to silanes and thiols, but are becoming of great practical interest because of their ability to produce SAMs on a range of metal oxide surfaces. Advantages of these SAMs are their higher hydrolytic stability under physiological conditions and the fact that no surface conditioning (i.e., acid treatment) is required to obtain high coverage. Therefore, importance of using phosphonic acids will be discussed in detail along with the supported results.

Secondly, the SAM layer using 11-aminoundecylphosphonic acid (11-AUPA) immobilized along with the use of γ-polyglutamic acid (γ-PGA) inducing apatite forming ability of the alloy was studied. Characterization of the apatite layer formed will be done using methods such as scanning electron microscopy (SEM), X-ray diffraction (XRD) study, X-ray Photoelectron Spectroscopy (XPS) etc.

Thirdly, this thesis would be focused on increasing the density of the apatite layer. Therefore, an approach utilized for this would be the use of sodium hydroxide (NaOH), which increases the -OH (hydroxyl) groups on the surface. This increase in -OH further improves the coverage and formation of SAM layer using 11-AUPA. Zinc-complex substitution technique will be used to determine the increased concentration of -OH groups on the alloy surface. In addition to this, results will be supported by the XRD, XPS, SEM etc.

In the second part of my work, the same method used above by using 11-AUPA immobilized along with the use of γ-PGA was applied to the ceramic biomaterials. Alumina ceramics are typically bioinert, meaning low ability of bone-bonding. Bioinert ceramics like alumina and zirconia maintain their physical and mechanical properties even in biological
environments. Therefore, in the final part of my thesis I will be implementing the methods discussed in the previous parts for increasing the bioactivity of ceramic material such as alumina.

References


**Tables and Figures**

**Table 1-1. Different type of some important biomaterials [1-5]**

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<th>Biomaterial System</th>
<th>Examples</th>
<th>Major type of application</th>
<th>Features</th>
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<td>Metallic</td>
<td>Steel-316</td>
<td>Structural</td>
<td>Biocompatible Good wear resistance</td>
</tr>
<tr>
<td></td>
<td>Co-Cr alloys: Co-Cr-Mo, Co-Cr-W-Ni, Co-Ni-Cr-Mo-Ti</td>
<td>Structural</td>
<td>Excellent biocompatibility Low strength-to-weight ratio</td>
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<td></td>
<td>Ti alloys: CP Titanium, Ti-6AL-4V, Ti-3Al-2.5V, Ti-6Al-7Nb</td>
<td>Structural</td>
<td></td>
</tr>
<tr>
<td>Ceramics</td>
<td>Alumina, Zirconia Hydroxyapatite, calcium phosphate Bioactive: Bioglass, Cervital</td>
<td>Structural and coatings</td>
<td>Bioinert Biodegradable Bioactive</td>
</tr>
<tr>
<td>Polymers</td>
<td>Polyethylene, silicone, UHMWPE, PVC</td>
<td>Structural</td>
<td>Bioinert Easy to manufacture Low cost</td>
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Figure 1-1. A bioactive bone bonding between a bioglass implant and its surrounding bone tissue [21].
Figure 1-2. The formation of SAMs on the substrate surface [63].
Chapter 2

THE STUDY OF 10-CARBOXYDECYLPHOSPHONIC ACID (10-CDPA) SELF-ASSEMBLED MONOLAYERS (SAMs) IN INDUCING APATITE FORMATION ON COBALT-CHROMIUM (Co-Cr) ALLOY

1. Introduction

Cobalt-Chrome (Co-Cr) is a metal alloy of cobalt and chromium categorized as mostly used base-metal alloy, which has been popularly known for its biomedical applications in the orthopedic and dental fields. The property such as high strength, ductility, malleability, and temperature endurance, pitting resistance, crevice corrosion resistance and wear resistance makes it an important biomaterial [1, 2]. When used for orthopedic implants it is usually composed of cobalt with chromium, molybdenum, and traces of other elements. Venable and Stuck realized the low level of corrosion of the cobalt based alloy vitallium by checking its electrolytic effect on surrounding tissue and bones [3]. Co-Cr alloys can be fabricated into different porous bodies to allow for mechanical fixation by tissue ingrowth. These alloys are well designed to replace bone and to be load bearing for an extended period, if not permanently. The importance of Co-Cr alloys can be understood by its use as the Austin Moore prosthesis and the Thompson prosthesis. The first-generation biologically fixed implants (i.e. porous-coated anatomic (PCA) and anatomic medullary locking (AML) implants) were manufactured of Co-Cr alloy. Still various modern prostheses are manufactured from this excellent alloy and are used in both cemented and porous forms for hip and knee replacement. In dentistry, Co-Cr alloys are commonly used with other composites like ceramics for partial dentures and recently have been used as metallic substructures for the fabrication of porcelain-fused-to-metal restorations and implant frameworks.
Biomedical application of Co-Cr is worldwide attracting much attention due to economical and physico-mechanical advantages. Even though there are various biomedical applications of using Co-Cr as outlined above, however there are still several biomedical challenges reported in clinical studies. Problems such as restenosis, release of inflammatory mediators and hypersensitivity have been reported when Co-Cr alloys are used in orthopedic and dentistry applications respectively [6, 7]. The biomedical challenges in case of orthopedic implants require immediate attention; especially post-surgical implant infection is an important problem where the infections occur on the implant surface [8-10]. The issues related to attachment of biomolecules such as microbes, proteins etc. and biofilm formation on the implant surface could lead to infections [11]. Result of these infections make patient to undergo two or more additional surgeries [12]. Infections occur as microorganisms are frequently introduced onto the implant surface during surgery and compete for the surface before tissue integration [13]. Improper tissue integration is another problem, which results in poor fixation of the bone tissue and the implant [14]. So, the surface of Co-Cr implant device becomes very important for existing applications and future applications. Therefore surface modification of the implants is also necessary, such as to enhance tissue integration, to remove problems related to loosening of implant and inflammations.

As we all know, bioactivity is one of the important requirements for biomaterials in the design of implantable devices. But Co-Cr alloys can’t bond with the natural bone directly, namely bio-inert materials [15, 16]. So our researches were mainly focused on improving the bioactivity of the Co-Cr alloys by introducing an active bone-like apatite layer onto their surfaces. By alkali treatment, Ti and its alloys can form apatite layer after soaking in simulated body fluid (SBF), which is a solution containing inorganic ion concentrations almost equal to those of human extracellular fluid [17-21]. But this modification is not suitable for Co-Cr alloys [22]. Self-assembled monolayer technique has been confirmed to modify the surface state by
using different biomolecules immobilization, such as modifying non fouling surfaces or bioactive surfaces [23]. It is well known that the growth rate of terminal group species when inducing apatite is in the following order: -PO₄H₂>-COOH>-CONH₂ approximately equal to -OH>-NH₂>-CH₃ approximately equal to 0 [24]. 10-carboxydecylphosphonic acid (10-CDPA: C₁₁H₂₃O₅P is an alkyl phosphonic acid derivative containing the carboxylic acid termination group. Zhang and co-workers have utilized a self-assembled monolayers of 10-CDPA on the surface of ZnO as a biosensor [25].

In this chapter, chemical surface modification by using 10-CDPA self-assembled layers was attempted to induce a bioactive surface on the Co-Cr alloy in vivo environment. As it already confirmed that alkaline pretreatment can improve the density of hydroxyl groups on the surface, which will be discussed in chapter 4, the alkaline pretreatment will also be used in these following experiments.

2. Experimental

2.1 Materials

The 10-carboxydecylphosphonic acid (10-CDPA) purchased from Dojindo Laboratories Co., Ltd. (Kumamoto, Japan) was used in our study. Chemical surface modification method was carried on the Co-Cr alloy substrate (Elemental mass content: 58% Co, 30% Cr, 6% Mo and 6% others) purchased from Nihon Shika Kinzouku Co., Ltd. (Osaka, Japan). Sodium dodecyl sulfate (SDS) solution, ethanol and acetone were all purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other reagents used in preparing the simulated body fluid were all purchased from Nacalai Tesque Inc. (Kyoto, Japan). Ultrapure water with a resistivity of 18 MΩ·cm was prepared using a water purification system (Direct-Q, Millipore Co., Billerica, MA, USA).
2.2 Formation of 10-CDPA acid monolayers

The Co-Cr alloy was cut into 15 mm diameter×1.0 mm specimens and polished (Buehler AutoMet 2 polishing machine) with #500, 1000 and 1500 waterproof silicon carbide papers. Then the polished specimens were cleaned by ultrasonic vibration followed in the order of 0.5% SDS aqueous solution, ethanol, acetone and ultrapure water for every 20 min. The cleaned alloy substrates above were denoted here as BC.

For the chemical pretreatment on the surface, the thin alloy substrates were immersed in 5 M NaOH aqueous solution under 60ºC for 48 h. Thus alkaline treated Co-Cr substrates were denoted here as NC. Then the BC samples and NC samples were rinsed with deionized water and dried. After that, all the samples were immersed in 5 ml of 1 M 10-CDPA ethanol solution at 36.5ºC for 2 days. Then the specimens were mildly rinsed in ethanol followed by ultrasonic irradiation in ultrapure water for 10 min and dried in the air. Thus modified samples by immersion in 10-CDPA acid were denoted here as BCM and NCM respectively.

2.3 SBF soaking treatment

In order to explore the bioactivity inducing ability of Co-Cr alloys by chemical surface modification, the samples were soaked in 30 ml of SBF and 1.5 SBF solutions for various periods of up to 28 days after the immersion dispose in 0.5 M and 1 M concentration of CaCl₂ solution from 1 to 2 days separately. The SBF and 1.5 SBF solutions were prepared according to the method proposed by Professor Kokubo [18]. The ion concentration of the SBF solution and those of the human blood plasma [26] are shown in Table 4-1. After SBF soaking treatment, the samples were immersed in ultrapure water for 1 day and then dried at room temperature.
According to the variables involved in our experimental process, we conducted our experiments by following the two main methods with the same parameters and procedure as showed in section 2.2. In the first method, all the specimens were treated with a fixed concentration of CaCl$_2$ (0.5 M) before soaking them into the SBF solutions. After CaCl$_2$ solution soaking, Co-Cr alloys were immersed in the SBF solutions for 7 days and 28 days. In the second method, all the specimens were treated with a fixed concentration of CaCl$_2$ (1 M) for 2 days before soaking them into the SBF solutions. Subsequently, the Co-Cr alloys were immersed in to 1.5 SBF and SBF solutions for 7 days. At the same time, some specimens were treated in the oven under 400°C for 1 h followed by the treatment of 1 M CaCl$_2$ solution. Then the Co-Cr alloys were immersed into SBF solutions for 7 days.

After SBF or 1.5 SBF soaking treatment, the samples were immersed in ultrapure water for 1 day and then dried at room temperature.

2.4 Surface characterization

The BC, NC, BCM and NCM samples were characterized by contact angle measurement (DMe-200, Kyowa Interface Science Co., Ltd., Saitama, Japan) and X-ray photoelectron spectroscopy (XPS; KRATOS AXIS-His, Shimadzu Co., Kyoto, Japan). The static contact angles toward distilled water were measured after 3 min which were the mean values of at least three different spots on each of the three different specimens. For the binding energies measured by XPS, they were corrected based on the binding energy of the C1s of the methylene groups of the hydrocarbon (284.6 eV) adsorbed onto the surface of the substrate in this study.

2.5 Surface structural features
The surface structure changes of the samples before and after SBF soaking treatment were characterized by thin-film X-ray diffraction (TF-XRD; MXP3V, Mac Science Ltd., Yokohama, Japan) and scanning electron microscopy (SEM; S-3500N, Hitachi Co., Tokyo, Japan). For TF-XRD, the angle of the incident beam was fixed at 1º against the surface of the samples and the measurements were performed using a step scanning mode with steps at 0.02º steps and 1s. For SEM observation, a thin film of gold was sputtered on the surface of the samples.

2.6 Assessment of adhesion using Scotch Tape

In order to evaluate the adhesion ability between the apatite and the substrates, Scotch Tape Test was used in this experiment. The main process was to cover the half surface of the samples by the tape and then peel it off gently. According to the surface structure changes of the samples before and after scotch tape test by SEM analysis we can judge the binding force of the apatite to the substrates.

3. Results and Discussion

3.1 Contact angle analysis

Figure 2-1 shows the contact angle images toward distilled water for Co-Cr alloys under different states. After the immersion in sodium hydroxide (NaOH) solution for 48 h, the contact angle of NC specimen was lower than the cleaned substrate (BC). After the immersion treatment of BC substrate by 1 M 10-CDPA solution, the contact angle also decreased obviously which demonstrated some new hydrophilic groups immobilized on the surface. These phenomena may be due to the reaction between -OH groups on the surface of BC substrate and 10-CDPA molecule resulting in final -COOH terminal groups which existed on the surface. Meanwhile, in
comparison with the BCM sample, the contact angle value decreased more after soaking in the 10-CDPA solution followed by NaOH pre-treatment. This change indicates the density of hydrophilic -OH groups can be improved by alkaline immersion.

3.2 XPS spectra analysis

Figure 2-2 shows high resolution XPS spectra for P2p and O1s peaks involved in surface treatment of BC samples with different solutions. The O1s photoelectrons for BC samples are located approximately at 532 eV and 530 eV, which is in agreement with the reported values for metal oxide (O²⁻) and hydroxide (OH⁻) species respectively [27]. Figure 2-2(a) is the normalized peak plot for O1s to clearly understand the effect of different solutions on the BC sample surface. It indicates the slight increment of metal oxide moieties on the surface after treatment with NaOH and 10-CDPA solution. However, the cumulative effect of both NaOH and 10-CDPA can be seen more effective with comparatively more amount of O²⁻ species. The O²⁻ species formed in the case of treatment with NaOH is clear indication of increase of µ-oxo due to formation of metal oxides and increased density of the dangling –OH groups on the bare Co-Cr surface [28-31]. O²⁻ species formed due to 10-CDPA treatment is attributed to P=O and P-OH [32] (which shows successful immobilization of 10-CDPA). The details of this influence of NaOH pretreatment would be discussed in Chapter 4. The surface when treated with NaOH and 10-CDPA together allows formation of denser immobilized layer of 10-CDPA, which is contributed by metal oxide formation using NaOH solution. Similarly, Figure 2-2(b) suggests the existence of P atom, showed by the binding energy peak at 133 eV after treatment with 10-CDPA, indicating the surface immobilization.

3.3 XRD and SEM analysis

-27-
Figure 2-3 shows the SEM image of the Co-Cr (BC) alloys without any treatment, after NaOH pretreatment (NC) and after NaOH pretreatment followed by the chemical treatment with 10-CDPA solutions (NCM). In comparison with the raw Co-Cr alloys, there are no changes after different chemical treatment. As this 10-CDPA SAMs layer is extremely thin, it’s undetectable by SEM images.

In the cases of the substrates by method 1, Figure 2-4 shows the XRD pattern and SEM images of the Co-Cr alloys pretreated with 0.5 M CaCl₂ solution for 1 day and soaked in SBF solutions for 7 days. After the treatment and immersion, there wasn’t any new peak except for the Co and α-CoCr peaks as detected by XRD analysis. At the same time, we can’t see any changes on the surface of the samples according to the SEM images. However, for the Co-Cr alloy samples immersed in SBF for 28 days with 0.5 M CaCl₂ solution pretreatment for 2 days, the SEM images show some new products formed on the surface. These products were confirmed to be apatite by XRD analysis in Figure 2-5. Although we can get the apatite formation after extending the soaking time in SBF to 28 days and some apatite particles combined tight to each other leading to the formation of a dense layer on the surface, these apatite layers formed were present only on the some part rather than covering the whole surface of Co-Cr alloy. These limited amount of apatite layer existence were clearly seen with the very weak peaks in the XRD patterns, as shown in Figure 2-5.

In the cases of the substrates by method 2, Figure 2-6 shows the XRD results and SEM images of the Co-Cr alloy samples immersed in 1.5 SBF for 7 days with 1 M CaCl₂ solution pretreatment for 3 days. From the XRD analysis, we can see there are new peaks at 26° and 32° except for the Co and α-CoCr peaks which indicated the apatite formed on the surface of Co-Cr alloys. We can observe some spherical particles formed on the surface clearly using SEM. But at the same time, the clear grinding marks under the apatite particles are still very visible, which
may indicate there is no apatite layer formed on the surface of these specimens. As these apatite particles have a very weak adhesion to the substrates, they are easily to peel off by the tape test experiments, as shown in Figure 2-6. These apatite particles may form due to the higher ion concentration, mainly of calcium and phosphorus in the 1.5 SBF solution and fell down to the surface.

Figure 2-7 shows the XRD results and SEM images of the Co-Cr alloy samples with 1 M CaCl$_2$ solution treatment for 2 days followed by immersion in SBF for 7 days. The XRD patterns clearly show the new and strong peaks at 26º and 32º which indicated the apatite formed on the surface of Co-Cr alloys. In comparison with the method 1, the apatite can form after soaking in SBF for 7 days by increasing the concentration of CaCl$_2$ solution to 1 M. But the apatite layer was not presented on the whole surface of the substrate and seemed to form discontinuous layer at the same time.

Figure 2-8 shows the XRD results and SEM images of the Co-Cr alloy samples with heat treatment under 400ºC for 1 h, immersed in SBF for 7 days with 1 M CaCl$_2$ solution pretreatment for 2 days. From the XRD patterns, we can clearly observe the new and strong peaks at 26º and 32º which indicated the apatite formed on the surface of Co-Cr alloys. The SEM images show a clear morphology of the apatite on the surface of the substrate. After heat treatment under 400ºC for 1 h, the apatite layer was presented to be a continuous and tightly bounded with the substrate.

4. Conclusions

In conclusion, self-assembled monolayers of the 10-CDPA was successfully immobilized on the surface of Co-Cr alloys in ethanol solution due to the dehydration/condensation reaction between the P-OH groups of the 10-CDPA and the OH groups on the surface of the alloy. This was confirmed by the formation of new peak of P atom as realized by XPS spectra analysis and
contact angle changes. But when the samples were treated with 0.5 M $\text{CaCl}_2$ solution in the case of method 1, the apatite was not easily and well induced although we extended the soaking time in SBF until 28 days. But in the case of method 2, with the increase in concentration of $\text{CaCl}_2$ solution from 0.5 M to 1 M, the formed apatite amount increased which indicated that relative high concentration of $\text{CaCl}_2$ solution is very necessary when the Co-Cr alloys treated with 10-CDPA SAMs. Besides, the heat treatment can improve the combination of the apatite layer with the substrate and should be researched more in the future. The results of this study therefore represent that the surface modification method by 10-CDPA could lead to the improvement of bioactivity behavior.
References


**Tables and Figures**

**Table 2-1** Ion concentration of simulated body fluid (SBF)

<table>
<thead>
<tr>
<th></th>
<th>Na(^+)</th>
<th>K(^+)</th>
<th>Ca(^+)</th>
<th>Mg(^{2+})</th>
<th>Cl(^-)</th>
<th>HCO(_3)(^-)</th>
<th>HPO(_4)(^{2-})</th>
<th>SO(_4)(^{2-})</th>
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<tr>
<td>Blood plasma</td>
<td>142</td>
<td>5</td>
<td>2.5</td>
<td>1.5</td>
<td>103.0</td>
<td>27.0</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>SBF</td>
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<td>2.5</td>
<td>1.5</td>
<td>148.8</td>
<td>4.2</td>
<td>1</td>
<td>0.5</td>
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<tr>
<td>1.5 SBF</td>
<td>213</td>
<td>7.5</td>
<td>3.75</td>
<td>2.25</td>
<td>223.2</td>
<td>6.3</td>
<td>1.5</td>
<td>0.75</td>
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</table>
Figure 2-1. Static contact angle (°) toward distilled water on the surface of Co-Cr specimen under different states.
Figure 2-2. XPS spectra of O1s and P2p on Co-Cr alloys before and after different treatments.
**Figure 2-3.** SEM images of the surfaces of Co-Cr alloy with and without NaOH and subsequent 10-CDPA treatments.
Figure 2-4. XRD patterns and SEM images of the surfaces of Co-Cr alloy before and after soaking in SBF for 7 days when treated with 0.5 M CaCl₂ solution.
Figure 2-5. XRD patterns and SEM images of the surfaces of Co-Cr alloy before and after soaking in SBF for 28 days when treated with 0.5 M CaCl$_2$ solution.
Figure 2-6. XRD patterns before and after soaking in 1.5 SBF solution and SEM images of the surfaces of Co-Cr alloy before and after tape test, which were soaked in 1.5SBF for 7 days when treated with 1 M CaCl$_2$ solution.
Figure 2-7. XRD patterns and SEM images of the surfaces of Co-Cr alloy after soaking in SBF and for 7 days when treated with 1 M CaCl₂ solution.
Figure 2-8. XRD patterns and SEM images of the surfaces of Co-Cr alloy after soaking in SBF and for 7 days when treated with 1 M CaCl₂ solution.
Chapter 3

THE STUDY OF 11-AMINOUNDECYLPHOSPHONIC ACID (11-AUPA) SELF-ASSEMBLED MONOLAYERS (SAMS) AND POLY-γ-GLUTAMIC ACID (PGA) IN INDUCING APATITE FORMATION ON COBALT-CHROMIUM (Co-Cr) ALLOY

1. Introduction

Co-Cr alloys have been used in a wide variety of biomedical applications, including dental, cardiovascular and orthopedic devices [1, 2]. Alloys of this type generally demonstrate high levels of biocompatibility and also possess excellent mechanical properties, including resistance to pitting, wear, high abrasion and crevice corrosion, as well as showing high fatigue strength, malleability and ductility [3, 4]. Co-Cr alloys are frequently used in implant materials during joint replacement procedures involving hard tissues, such as tooth and bone, where they come into direct contact with blood, soft tissue and bone [5, 6]. Despite their many advantages and widespread application in biomedical devices, several issues have been reported with regard to the use of Co-Cr alloys in clinical studies, most notably a lack of bioactivity, leading to poor bone bonding.

An important requirement for a material to demonstrate bioactivity is the ability to form biologically active bone-like apatite on its surface [7]. The formation of apatite can even occur in simulated body fluid (SBF), which is a solution containing inorganic ion concentrations almost equal to those of human extracellular fluid [8, 9]. In a physiological environment, chemical surface modification processes such as alkali treatment and anodic oxidation can be used to convert Ti and its alloys [10-14] and Ta [15] into bioactive materials. However, the use of these
modifications has been shown to be ineffective for Co-Cr alloys [10]. Although the application of a bioactive glass coating to the surface of Co-Cr alloys has been evaluated in detail [16], very few studies have been reported pertaining to the precipitation of apatite onto Co-Cr alloys in SBF.

It was recently reported that self-assembled monolayers (SAMs) can be successfully constructed on the surfaces of a variety of different metals, including Co, Co-Cr alloys, stainless steel, gold and Ti-45Nb alloy [17-21]. And we also confirmed that 10-CDPA phosphonic acid can be immobilized to the Co-Cr alloys as a SAM layer in the last chapter. Although the apatite layer could be induced by using 10-CDPA phosphonic acid SAM layer, the apatite layer showed either partial coverage of the substrates or discontinuous morphology. In addition, our group recently demonstrated that cross-linked γ-polyglutamic acid (γ-PGA) can be used to induce bone-like apatite layers in SBF due to big amount of carboxyl groups [22-24]. Taken together, these findings suggest that the immobilization of γ-PGA onto a Co-Cr alloy constructed from SAMs could be used to increase the bioactivity of the alloy.

In this chapter, we have prepared a series of SAMs based on 11-aminoundecylphosphonic acid ((11-AUPA):NH$_2$(CH$_2$)$_{11}$PO$_3$H$_2$·HBr) and subsequently immobilized γ-PGA. The apatite-forming properties of these layers were subsequently assessed in SBF. Then we also incorporate the Ca$^{2+}$ into the specimens to enhance their apatite-forming ability and studied the influence of calcium chloride (CaCl$_2$) concentration on apatite precipitation by this method above in a simulated body fluid. Finally, the effect of the addition of N-Hydroxysuccinimide (HOSu) when preparing the mixed PGA solution on the final apatite formation was also studied.

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2. Experimental

2.1 Materials

The Co-Cr alloy used in the current study was purchased from Nihon Shika Kinzoku Co., Ltd. (Osaka, Japan), and its elemental composition is listed in Table 3-1. 11-aminoundecylphosphonic acid (11-AUPA) was purchased from Dojindo Laboratories Co., Ltd. (Kumamoto, Japan). Cross-linked γ-polyglutamic acid (γ-PGA) with a molecular weight in the range of 800 to 1200 kDa was purchased from Meiji Seika Kaisha, Ltd. (Tokyo, Japan). N-Hydroxysuccinimide (HOSu) was purchased from Wako Pure Chemical Industries (Osaka, Japan) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All of the other reagents used in the current study were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Ultrapure water with a resistivity of 18 MH·cm was prepared using a water purification system (Direct-Q, Millipore Co., Billerica, MA, USA).

2.2 Formation of 11-AUPA self-assembled monolayers and γ-PGA monolayers

According to the study factors mentioned above, the specimens will be prepared and researched by the following two methods. In both two methods, all the specimens were prepared with the same process before chemical surface modification. The Co-Cr alloys were cut into thick disks (15 mm diameter×1.0 mm), which were polished with #500, 1000 and 1500 SiC waterproof abrasive papers. The polished disks were then subjected to ultrasonic irradiation in a 0.5% sodium dodecyl sulfate aqueous solution, followed by ethanol and then acetone for 20 min each. In the first method, the chemical surface modification was performed by soaking the disks in 5 mL of a 1 M 11-AUPA solution in ethanol at 36.5°C for 2 days, followed by soaking in an
aqueous solution of γ-PGA with stirring for 1 day at room temperature. The reagent composition of the γ-PGA solution is listed in Table 3-2.

2.3 SBF soaking treatment

In order to explore the bioactivity inducing ability of Co-Cr alloys by using 11AUPA+PGA modification method, the untreated and treated specimens were subsequently immersed in CaCl$_2$ solutions of various concentrations including 0.01 M, 0.1 M, 0.5 M and 1 M at 36.5°C for 1 day, named as method 1. In the another method, the chemical surface modification was performed in the way as the first method besides the differences when preparing the mixed PGA solution without adding HOSu and the defined 0.5 M CaCl$_2$ solutions treatment, named as method 2. Finally, all the specimens treated in both methods were soaked in 30 mL of SBF (Na$^+$: 142.0, K$^+$: 5.0, Mg$^{2+}$: 2.5, Cl$^-$: 147.8, HCO$_3^-$: 4.2, HPO$_4^{2-}$: 1.0, and SO$_4^{2-}$: 0.5 mM) at 36.5°C for 7 days [8]. After soaking, the disks were removed from the SBF and immersed in ultrapure water for 1 day to remove any excess residual water-soluble salts before being dried at room temperature.

2.4 Surface characterization

All the samples were characterized by contact angle measurement (DMe-200, Kyowa Interface Science Co., Ltd., Saitama, Japan) and X-ray photoelectron spectroscopy (XPS; KRATOS AXIS-His, Shimadzu Co., Kyoto, Japan). The static contact angles toward distilled water were measured after 3 min which were the mean values of at least three different spots on each of the three different specimens. For the binding energies measured by XPS, they were corrected based on the binding energy of the C1s of the methylene groups of the hydrocarbon (284.6 eV) adsorbed onto the surface of the substrate in this study.
2.5 Surface structural features

The surface structure changes of the samples before and after SBF soaking treatment were characterized by thin-film X-ray diffraction (TF-XRD; MXP3V, Mac Science Ltd., Yokohama, Japan) and scanning electron microscopy (SEM; S-3500N, Hitachi Co., Tokyo, Japan) combined with the energy-dispersive X-ray microanalyzer (EDX; EMAX Energy, Horiba Ltd., Kyoto, Japan). For TF-XRD, the angle of the incident beam was fixed at 1º against the surface of the samples and the measurements were performed using a step scanning mode with steps at 0.02º steps and 1s. For SEM observation, a thin film of gold was sputtered on the surface of the samples.

3. Results and Discussion

3.1 Contact angle analysis

Firstly, the inducing apatite ability of Co-Cr alloys was studied by method 1 which used 1 M CaCl₂ solution treatment with a following immersion in SBF for 3 and 7 days.

Contact angle images were recorded for the Co-Cr alloy with ultrapure water both before and after it had been soaked in a solution of 11-AUPA (Fig. 3-1). Contact angle images were also recorded in the same way following the soaking of the 11-AUPA-treated material in a solution of γ-PGA (Fig. 3-1). The results of these experiments demonstrated that the contact angle decreased from 73.6° to 54° and then to 24.4° after the 11-AUPA and γ-PGA treatments, respectively, which indicated that the surface of the alloy was becoming increasingly hydrophilic with each treatment.

Secondly, we examined the necessity of HOSu composition in the mixed PGA solution in inducing the final formation of apatite by using method 2. Figure 3-2 shows the static contact angles (º) toward distilled water on the surface of Co-Cr alloy under different states. In comparison with the contact angle of the samples after soaking in the 11-AUPA solution, in both
cases of the samples under the subsequent treatment of PGA mix solution without and with HOSu composition, the contact angles all decrease significantly. But we can also discover that the contact angle decrease more after soaking in the PGA solution with addition of HOSu, which shows a more hydrophilic surface. According to the analysis in the following analysis of XPS spectra, this hydrophilic performance change was due to the precipitation of γ-PGA biomacromolecule, which containing abundant of -COOH groups.

3.2 XPS spectra analysis

In order to have a more understanding of the changes in the chemical compositions of the samples, the XPS spectra analysis was also used.

In the case of method 1, the XPS spectra of the O1s, C1s, N1s and P2p regions of the Co-Cr alloys both before and after the treatment of the alloys with 11-AUPA are shown in Figure 3-3. Notably, there was a significant increase in the intensity of the peak around 285 eV in the C1s spectrum following the treatment of the Co-Cr alloy with 11-AUPA. The O1s spectrum of the untreated alloy contained peaks at 530 eV and 532 eV, which were attributed to the metal oxide and hydroxide moieties, respectively [25]. After being soaked in a solution of 11-AUPA, the XPS spectrum of the Co-Cr alloy contained a large peak at 531 eV, which was attributed to the P-OH or P=O bonds [26]. The spectrum of the 11-AUPA-soaked material also contained two small peaks around 414.8 eV and 133 eV in the N1s and P2p regions, respectively. These results therefore suggested that 11-AUPA had been successfully immobilized onto the surface of the Co-Cr alloy.

In the case of method 2, the main element of the C1s and O1s were characterized by XPS, as shown in Figure 3-4. After soaking in the PGA mixed solution without and with HOSu composition, there appears a new peak at about 288.5 eV according to the details C1s region spectrum, which were resulted from the new functional groups -COOH in the PGA
macromolecule. However, with the addition of the HOSu into the mixed PGA solution, the intensity of the C1s peaks increased, which indicated more content of PGA were attached to the surface of the Co-Cr alloy. Meanwhile, the position of the O1s peak also moved to the left clearly due to the immobilization of PGA macromolecule. And this effect leads to a more moving position of it in the case of samples treated with addition of HOSu composition. Therefore, together with the contact angle changes in Figure 3-2, these results suggested that HOSu could successfully increase the final amount of the PGA combined to the substrate’s surface.

### 3.3 XRD and SEM analysis

In the case of method 1, SEM images of the Co-Cr alloys before and after their sequential treatment with 11-AUPA and γ-PGA are shown in Figure 3-5. Significant differences were not observed even after the treatments. The TF-XRD patterns of the Co-Cr alloys were measured before and after their sequential treatment with 11-AUPA, γ-PGA and a 1 M solution of CaCl$_2$ in SBF for 3 or 7 days, and the results are shown in Figure 3-6. The TF-XRD pattern of the untreated Co-Cr alloy in the $2\theta$ mode contained peaks at $43^\circ$ and $47^\circ$, which were assigned to Co-Cr and $\alpha$-Co, respectively. No changes were observed in the positions of these peaks after the different soaking treatments. However, two new broad peaks appeared at $26^\circ$ and $32^\circ$ in the $2\theta$ mode for the Co-Cr alloys treated with 11-AUPA and γ-PGA after they had been soaked in SBF for 3 days. SEM images were collected for the Co-Cr alloys before and after their sequential treatment with 11-AUPA, γ-PGA and a 1 M solution of CaCl$_2$ in SBF for 3 or 7 days, and the results are shown in Figure 3-7. The results revealed that a layer of fine particles formed in the alloys treated with CaCl$_2$ in SBF that almost covered their entire surface. This effect was not observed in the untreated samples.
Meanwhile, we also examined the effect of the different CaCl\textsubscript{2} concentration on the formation of apatite with an immersion treatment in SBF for 4 and 7 days. The TF-XRD patterns of the Co-Cr alloy were recorded following their sequential treatment with 11-AUPA, \(\gamma\)-PGA and different concentrations of CaCl\textsubscript{2} in SBF for 4 and 7 days, and the results are shown in Figure 3-8. No matter in the cases of soaking in the SBF for 4 days or 7 days, obvious broad peaks corresponding to low-crystalline apatite appeared at 26° and 32° in the 2θ mode were observed in the samples treated with 0.5 M and 1 M solutions of CaCl\textsubscript{2}. However, in the cases of samples treated with 0.01 M and 0.1 M solutions of CaCl\textsubscript{2}, there are no obvious peaks were observed except the peaks of Co-Cr and \(\alpha\)-Co at 43° and 47° respectively, which suggested no apatite formed on the surface even longing the soaking time in SBF from 4 days to 7 days. This phenomenon indicated that incorporation of Ca\textsuperscript{2+} ions into the alloy with \(\geq0.5\) M solution of CaCl\textsubscript{2} was very necessary to allow for the formation of apatite on the alloy.

Figure 3-9 shows the SEM images of the Co-Cr alloys when treated with different concentration of CaCl\textsubscript{2} solutions after soaking SBF for 4 and 7 days respectively. In accordance with the results of TF-XRD patterns (Fig. 3-8), there are hardly changes before and after soaking in SBF for 4 and 7 days when the samples treated with 0.01 M and 0.1 M concentration of CaCl\textsubscript{2} solutions (Fig. 3-9-1, Fig. 3-9-2 ). Nevertheless, we can clearly discover the new products prepared on the surface of the Co-Cr alloys when the samples treated with 0.5 M and 1 M concentration of CaCl\textsubscript{2} solutions (Fig. 3-9-3, Fig. 3-9-4). The new product, confirmed to apatite by the TF-XRD results above, includes two different states which is one layer of small and fine spherical particles and some relative big particles gathered by some little apatite spherical particles. At the same time, we can see the size of the apatite particles which both constituted the layer and the relative particles increasing obviously and the amount of the big particles above the apatite layer also increased when longing the soaking time in SBF from 4 days to 7 days. On the other hand, we found that the small spherical particles consisted in the apatite layer became
bigger when increasing the concentration of CaCl₂ solution under same soaking days in SBF. This phenomenon may suggest 1 M CaCl₂ solution treatment has a better apatite-forming ability than 0.5 M CaCl₂ solution treatment. However, in the case of 1 M CaCl₂ solution treatment experiment, although we can obtain the apatite layer covering the substrate finally, in the meantime we found the SBF solution became a little turbid in comparison with the original clear SBF solution. This phenomenon may due to the inhomogeneous apatite nucleation by high concentration of CaCl₂ solution treatment when samples soaked in the SBF solution, which leading to the apatite produced in the solution and fell down to the surface. As this kind of apatite has no combination force with the substrate, and will also consume the amount of the compositions exist in the SBF solution for formatting the apatite on the surface and affect the SBF solution, we should control the concentration of CaCl₂ solutions in this method. But this effect didn’t happen in the case of 0.5 M CaCl₂ solution treatment. So from this aspect, 0.5 M CaCl₂ solution treatment plays a better role in this method.

In the case of method 2, Figure 3-10 and Figure 3-11 shows the TF-XRD patterns and SEM images of the Co-Cr alloys when treated without the addition of HOSu in the PGA solutions after soaking in SBF for 4 and 7 days respectively. The TF-XRD patterns shows there are no changes with the treatment of method 2 even after soaking in SBF for 7 days. However, there appeared some white particles on the surface. These particles were agglomerated by some little spherical particles with a size range of 1 μm, which was similar to the products precipitated on the surface of Co-Cr alloy by method 1. As the amount of these new deposits is limited, we can’t get the relative analysis from the XRD results. In order to confirm these particles, the EDX analysis was used, as shown in Figure 3-12. Except the elements of Co, Cr, Mo, O and C contained in the substrate, the Ca and P element peaks were also detected on the surface according to the EDX spectra with a ratio of 1.67, which suggested that these deposits were hydroxyapatite.
According to the design of our experiments, the amount of the final PGA macromolecule attached to the surface will dominate the possibility and quantity of the apatite produced on the surface. In the case of the samples treated with the PGA solution without the addition of HOSu, although there are some PGA immobilized onto the surface, the intensity is not strong enough to induce the huge mass apatite with a totally coverage formed onto the surface. Therefore, the HOSu is very important in successful apatite induction.

These results indicate that γ-PGA had been successfully immobilized on the Co-Cr alloy following the sequential soaking of the alloy with a solutions of 11-AUPA and γ-PGA solution containing EDC·HCl and HOSu. Alginate can be immobilized on stainless steel surfaces that have been modified with 3-aminopropyltriethoxysilane via the formation of amide bonds [27]. With this in mind, it was assumed that the γ-PGA used in the current study was also being immobilized on the surface of the Co-Cr alloy, albeit through the formation of amino bonds, as shown schematically in Figure 3-13. Namely, the 11-AUPA reacted with the Co-Cr alloy via a dehydration/condensation reaction between the P-OH groups of the 11-AUPA and the OH groups on the surface of the alloy. This result was confirmed by XPS analysis, which revealed the presence of N and P atoms derived from 11-AUPA after the treatment process (Fig. 3-3). When the 11-AUPA-treated alloy was subsequently immersed in a solution of γ-PGA containing HOSu and EDC·HCl, the amino groups of the 11-AUPA reacted with the carboxyl acid groups in γ-PGA to form the corresponding amide bond. The occurrence of this reaction was supported by the observed change in the contact angle of the alloy (Fig. 3-1). The formation of the SAMs was based on the naturally occurring -OH groups on the surface of the alloy. And the final induction ability was decided by the high density of 11-AUPA SAM layers and final amount of PGA attached to the surface of Co-Cr alloy by the reaction with 11-AUPA SAM layers. If the density of the -OH groups on the surface of the alloy could be increased with a pre-treatment process, then we would expect that the amount of immobilized 11-AUPA and γ-PGA on the
surface of the alloy would also increase, which would consequently lead to an increase in the formation of apatite. The effect of the pre-treatment process on the reactivity of the alloy will be examined in greater detail as part of our future work.

The modification process described here allowed for the formation of apatite on the surface of a modified Co-Cr alloy in a simulated body environment. The mechanism of apatite formation on the Co-Cr alloy involved the formation of chemical bonds on the surface of the alloy between 11-AUPA and γ-PGA. The carboxyl groups on the γ-PGA units would interact with the Ca\(^{2+}\), HPO\(_4^{2-}\) and PO\(_4^{3-}\) ions to allow for the heterogeneous nucleation of the apatite in SBF. Furthermore, sufficient Ca\(^{2+}\) ions would be released from the surface of the specimens into the SBF, leading to an increase in the degree of supersaturation with respect to the apatite when the sample was treated with an aqueous solution of 0.5 M and 1 M CaCl\(_2\).

4. Conclusions

SAMs functionalized with 11-AUPA were formed on the surfaces of Co-Cr alloys in aqueous solution. γ-PGA was then immobilized on the SAMs via a covalent cross-linking reaction. Apatite layers subsequently formed on the surfaces of the modified alloys when they were submerged in SBF containing a sufficient amount of Ca\(^{2+}\). HOSu plays an important role in final apatite induction. The results of this study therefore represent a novel surface modification method that can be used to increase the bioactivity of bioinert materials.
References


### Tables and Figures

#### Table 3.1 Chemical Composition of Co-Cr alloy

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<tr>
<th>Elements</th>
<th>Co</th>
<th>Cr</th>
<th>Mo</th>
<th>Others</th>
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<td>58</td>
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#### Table 3.2 Reagents composition of PGA

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<tr>
<td>γ-PGA</td>
<td>2.0</td>
</tr>
<tr>
<td>EDC·HCl</td>
<td>3.1</td>
</tr>
<tr>
<td>HOSu</td>
<td>1.8</td>
</tr>
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</table>
Figure 3-1. Contact angle images for Co-Cr alloys before and after chemical surface modification.
Figure 3-2. Contact angle images for Co-Cr alloys before and after chemical surface modification.
Figure 3-3. XPS spectra of O1s, C1s, N1s and P2p on Co-Cr alloys before and after treatment with 11-AUPA solution.
Figure 3-3. (Continued)
Figure 3-4. XPS spectra of O1s and C1s on Co-Cr alloys before and after treating with PGA solution with and without HOSu composition.
**Figure 3-5.** SEM images of the surfaces of Co-Cr alloy with and without 11-AUPA and subsequent γ-PGA treatments.
Figure 3-6. TF-XRD patterns of the surfaces of Co-Cr alloy with and without 11-AUPA, γ-PGA and 1 M-CaCl₂ treatments, which were all soaked in SBF for 3 and 7 days.
Figure 3-7. SEM images of the surfaces of Co-Cr alloy with and without 11-AUPA, γ-PGA and 1 MCaCl₂ treatments, which were all soaked in SBF for 3 and 7 days.
Figure 3-8. TF-XRD patterns of the surfaces of Co-Cr alloy treated with various concentrations of CaCl$_2$, which were soaked in SBF for 4 and 7 days.
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Chapter 4
THE INFLUENCE OF NaOH PRETREATMENT ON INDUCING APATITE FORMATION ON 11-AUPA-MODIFIED COBALT-CHROMIUM (Co-Cr) ALLOYS IN THE SIMULATED BODY FLUID

1. Introduction

As cobalt-chromium (Co-Cr) alloys possess a good combination of corrosion resistance, mechanical properties and biocompatibility, they have been used as biomaterials for orthopedic, dental, neurological and cardiovascular implant devices [1-3]. From a long-term perspective of longevity of the implanted biomaterials, Co-Cr alloys are preferred due to their particularly excellent wear resistance in comparison with other metallic biomaterials such as titanium alloys and stainless steels [4-8]. However, they have one fatal shortcoming in direct bonding with natural bone and therefore classified as bio-inert materials [9-12]. In order to overcome this shortcoming, some researchers have tried to make bioactive ceramic coatings on Co-Cr alloys before implanted into the body environment [13].

The most common way to improve their bioactivity is to fabricate an active bone-like apatite layer on the surface of Co-Cr alloys. In previous work, many researchers tried different kinds of techniques in manufacturing bioactive composite materials based on Co-Cr alloys. Hydroxyapatite (HA) is the main structural component of human bone that could be applied in biomedical applications as a coating layer [14, 15]. Although it was confirmed that plasma-sprayed method, sol-gel method or electrolytic deposition could create such bioactive Co-Cr or other metal based composites [16-18], a major problem of these implants is the weak
physisorption of macromolecules or adhesion promoting additives between HA layer and Co-Cr substrates. If the Co-Cr alloys and apatite could be bonded by chemical force as one composite material, the above problem will be solved.

Some chemical surface modification method such as alkali treatment has been used in improving the bioactivity of titanium and its alloys and tantalum materials [19-21]. However this method is not suitable for Co-Cr alloys. But it was recently demonstrated that self-assembled monolayers (SAMs) can be successfully immobilized onto the surface of Co-Cr alloys and aluminium materials by soaking these materials into some organic phosphorous acid [22]. Surface modification of Co-Cr alloy using self-assembled monolayers (SAMs) could act as a precursor for further modification of the alloy surface. A thin film of SAM with typical thickness in the range of 1-3 nm [23, 24] covers the implant surface uniformly and resists it from biological surroundings. The ease of modification through the selection of appropriate terminal functional groups has motivated the research utilizing SAMs for potential medical applications [25-29]. Alkanethiols, [30, 31] disulphides, [32] trichlorosilanes, [32, 33] trimethoxysilanes, [34] organosilicon hydrides, [34] phoshonic acids, [33, 35] phosphates, [36] hydroxamic acid [37] and carboxylic acid [38] are some of the popularly utilized SAMs on wide range of substrates [24, 32, 39, 40].

The purpose of the present study is to induce hydroxyapatite generation with an improved binding strength to the substrates in SBF by chemical surface modification method. The immobilization of suitable high-density terminal group species with a good apatite inducing ability in the simulated body fluid (SBF) is especially important for adhesion promotion on Co-Cr substrates. Our group had already researched that the bioactivity could be induced by the immobilization of cross-linked γ-polyglutamic acid (γ-PGA) self-assembled monolayers on the Co-Cr alloys [41]. So in the following research, the effects of NaOH pretreatment on the density
of the -OH surface functional groups and the binding force analysis between induced apatite and Co-Cr alloys will be done.

2. Experimental

2.1 Materials

The 11-aminoundecylphosphonic acid purchased from Dojindo Laboratories Co., Ltd. (Kumamoto, Japan), γ-PGA with a molecular weight in the range of 800 to 1200 kDa purchased from Meiji Seika Kaisha, Ltd. (Tokyo, Japan), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and N-Hydroxysuccinimide (HOSu) purchased from Wako Pure Chemical Industries (Osaka, Japan) were used in our study. Chemical surface modification method was carried on the Co-Cr alloy substrates (Elemental mass content: 58% Co, 30% Cr, 6% Mo and 6% others) purchased from Nihon Shika Kinzouku Co., Led. (Osaka, Japan). Sodium dodecyl sulfate (SDS) solution, ethanol, acetone, ammonium chloride, zinc chloride and 30 vol% ammonium hydroxide solution, nitric acid solution were all purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other reagents used in preparing the simulated body fluid were all purchased from Nacalai Tesque Inc. (Kyoto, Japan). Ultrapure water with a resistivity of 18 MΩ·cm was prepared using a water purification system (Direct-Q, Millipore Co., Billerica, MA, USA).

2.2 Formation of 11-AUPA self-assembled monolayers and γ-PGA monolayers

The Co-Cr alloy was cut into 15 mm diameter×1.0 mm specimens and polished (Buehler AutoMet 2 polishing machine) with #500, 1000 and 1500 waterproof silicon carbide papers. Then the polished specimens were cleaned by ultrasonic vibration in the following order of 0.5%
SDS aqueous solution, ethanol, acetone and ultrapure water for every 20 min. The cleaned alloy substrates above were BC.

In order to study the influence of the alkaline pretreatment on the density of surface functional groups on the surface, the thin alloy substrates were immersed in 5 M NaOH solution with a shaking speed of 120 r/h under 60°C for 48 h. Thus alkaline treated Co-Cr substrates NC. Then the BC samples and NC samples were immersed in 5 ml of 1 M 11-AUPA ethanol solution at 36.5°C for 2 days with a following mildly rinse in ethanol, ultrasonic irradiation in ultrapure water for 10min and dried in the air. Thus modified samples by immersion in 11-AUPA acid were BCM and NCM respectively. Then all the samples were soaked in an aqueous mixed solution containing 2.0% (Mass content) γ-PGA, 3.1% EDC·HCl and 1.8% HOSu under stirring for 1 day at room temperature.

2.3 SBF soaking treatment

In order to explore the bioactivity inducing ability of Co-Cr alloys by chemical surface modification, the samples were soaked in 30ml of SBF (Na+:142.0, K+:5.0, Mg²⁺: 2.5, Cl⁻: 147.8, HCO₃⁻: 4.2, HPO₄²⁻: 1.0, and SO₄²⁻: 0.5 mM, PH7.40) at 36.5°C for various periods of up to 7 days after the immersion dispose in 1 M CaCl₂ solution. After SBF soaking treatment, the samples were immersed in ultrapure water for 1 day and then dried at room temperature.

2.4 Surface characterization

The BC, NC, BCM and NCM samples were characterized by contact angle measurement (DMe-200, Kyowa Interface Science Co., Ltd., Saitama, Japan). The static contact angles toward distilled water were measured, which were the mean values of at least three different spots on each of the three different specimens. After NaOH pretreatment, the samples were characterized
by Fourier transform infrared spectrometer (FT/IR; FT/IR-6100; JASCO Co., Kyoto, Japan) using an interferometer scanning rate of 2 mm/s and resolution of 4 cm\(^{-1}\).

A zinc-complex substitution technique was used to determine the concentration of active hydroxyl groups on the surface of BC and NC samples. The process of the zinc-complex substitution technique is showed in Figure 4-1. The BC samples were soaked in a 5 M NaOH solution at 60º under a 120 r/h for 48 h in a desktop shaking water bath (Personal H-10 incubator, TAITEC, Japan). And then the BC samples and NC samples were soaked in 8 ml mixed solution of ammonium chloride, zinc chloride and ammonium hydroxide solution for 5 mins. The preparation process of the mixed solution is listed in Figure 4-2. After soaking in the mixed solution, all the samples were immersed in ultrapure water for 10 mins by ultrasonic vibration and dried in a desiccator for 1 h. Then they were soaked in 5 ml of 2.42 M nitric acid for 10 mins. The concentration of zinc ions released from the zinc complex to the solution was measured by inductively coupled plasma optical emission spectrometry (ICP-OES; Optima 4300DV CYCLON, Perkin-Elmer Inc., London, UK). And the concentration of active hydroxyl groups on the surface of Co-Cr alloys named \(C_{OH}\) (number/nm\(^2\)) was calculated by the following equation [42]:

\[
C_{OH} = \left\{ \left( \frac{C_{Zn} \times 10^{-6} \times V \times A}{M \times S} \right) \right\} \times 2 \quad (1)
\]

In the above equation, \(C_{Zn}\) is the concentration of zinc ions (ppb) measured by ICP, \(V\) is the volume of 2.42 M nitric acid (L), \(A\) is Avogadro’s number (6.02\(\times\)10\(^{23}\)), \(M\) is the molecular weight of zinc (62.75), and \(S\) is the surface area of samples (nm\(^2\)).

2.5 Surface structural features

The surface structure changes of the samples before and after SBF soaking treatment were characterized by thin-film X-ray diffraction (TF-XRD; MXP3V, Mac Science Ltd.,
Yokohama, Japan) and scanning electron microscopy (SEM; S-3500N, Hitachi Co., Tokyo, Japan). For TF-XRD, the angle of the incident beam was fixed at 1° against the surface of the samples and the measurements were performed using a step scanning mode with steps at 0.02° steps and 1s.

2.6 Scotch Tape test

In order to evaluate the adhesion ability between the apatite and the substrates, Scotch Tape Test was used in this experiment. The main process was to cover the half surface of the samples by the tape and then peeled it off gently. According to the surface structure changes of the samples before and after scotch tape test by SEM analysis, we can judge the binding force of the apatite to the substrates.

3. Results and discussion

3.1 Contact angle analysis

Figure 4-3 shows the contact angle analysis results. The wettability was checked using distilled water for Co-Cr alloys under different states. In comparison with the bare Co-Cr alloy, the sodium hydroxide (NaOH) pretreated alloy surface exhibits decreased contact angle from 73.6° to 55.1° owing to the improved wettability as shown in Figure 4-3(b). The similar change in contact angle value to 53.8° was observed after 11-AUPA treatment also, indicating enhanced hydrophilicity as shown in Figure 4-3(c). This is obvious as 11-AUPA treatment leads to formation of some new hydrophilic groups such as –COOH immobilized on the Bare Co-Cr alloy surface. The 11-AUPA treatment on the alloy surface with NaOH pretreatment, which was pretreated with NaOH solution further seems to have improved wettability with least contact angle value of 39.6° as shown in Figure 4-3(d). This improvement in wettability indicates that the
density of the self-assembled monolayers of 11-AUPA acid was improved by alkaline immersion.

### 3.2 FT/IR analysis

FTIR spectra of the samples were prepared by removing the surface oxide layer completely. Figure 4-4 shows the FT-IR spectra of the BC and NC alloy samples prepared in the current study before soaking in 11-AUPA solution. It shows significant difference due to the reaction with NaOH solution as new peaks were clearly seen at 1540 cm$^{-1}$ and 890 cm$^{-1}$ in the NC samples. These peaks are assigned to the Na-O bond by the Rao et al [43]. Further existence of the Na-O bond is strongly supported by William et al [44], as they have studied the reactions of different metals with NaOH solutions, which shows the formation of Na$_2$O and Metal oxide (or sodium metalate). The very strong and broad peak appeared at 890 cm$^{-1}$ was assigned to the product of sodium chromate [45]. In addition, the NaOH pretreatment resulted in an obvious increase of the peak which is observed at 3730 cm$^{-1}$ assigned to the -OH dangling bond, which indicates the density of the -OH groups can be improved by chemical surface treatment [46].

### 3.3 ICP analysis

Table 4-1 shows the concentration of zinc ions (ppb) measured by ICP. Here three samples were used and averaged before calculating the final density of the hydroxyl groups on the surface of the BC and NC samples. According to the data showed in the table 1, we calculated the intensity of the hydroxyl groups on the surface of BC and NC samples separately by equation 1. The density of -OH groups on the surface after NaOH treatment increased from 0.190 (number/nm$^2$) to 0.846 (number/nm$^2$). This increase of -OH groups also supports the increase in wettability as checked by contact angle analysis. Further Sakamoto et al [42]
demonstrated the effect of density of -OH groups, according to which shear bond strength between SAM layer and substrate increases with higher density of -OH groups on the surface.

3.4 XRD analysis

The apatite formation after 11-AUPA treatment and soaking with SBF solution for both untreated and NaOH treated alloy samples was confirmed by the thin film XRD (TF-XRD) pattern obtained as shown in Figure 4-5. The pattern obtained for untreated Co-Cr alloy shows 2θ peak at 43º and 47º, which were assigned to Co-Cr and α-Co, respectively. This pattern thus changed after the alloy was soaked in SBF solution for 4 and 7 days as shown in Figure 4-5, and new peaks at 26º and 32º were appeared, which were assigned to apatite. The increase in intensity from 4 days to 7 days indicates formation of more amount of apatite. Similar confirmation was obtained after NaOH solution treatment also as shown in Figure 4-5.

3.5 SEM analysis

The Figure 4-6 shows the SEM result obtained for the Co-Cr alloy surface with and without the NaOH treatment. The apatite layer, which is formed after soaking the untreated alloy in SBF solution for 4 days, shows formation of less dense and separated spherical particles as clearly seen in Figure 4-6 (b). In fact, these spherical particles grow in size when alloy further kept in solution for 7 days as can be seen in Figure 4-6 (c), however still the density of the particles formed is not good. Therefore, a pretreatment using NaOH solution is done and SEM morphology thus obtained in Figure 4-6 (e) & (f), clearly indicates the formation of dense apatite layer on the surface.

3.6 Tape test
The scotch tape test demonstrates the increased binding strength between apatite layer and the Co-Cr alloy substrate. The result obtained when the alloy is kept in SBF for 7 days, after performing the test is shown in Figure 4-7. The untreated alloy surface as shown in Figure 7a seems to be etched easier, as smooth apatite layer surface before the tape test transforms into a rough etched surface when seen through SEM. However, there is almost no effect on the NaOH pretreated apatite layer as shown in Figure 4-7 (b). The surface morphology doesn’t change when seen at the same scale. The tape test result obtained indicates the formation of dense apatite layer as confirmed from SEM analysis in Figure 4-6, after pretreatment using NaOH solution. The dense layer thus formed after NaOH treatment allows the increased binding strength between the particles and also with the alloy surface.

4. Conclusions

In conclusion, we have successfully increased the bioactivity of the Co-Cr alloys using the treatments. Our results show a synergistic effect of using NaOH and 11-AUPA treatments to increase the density and binding strength of the apatite layer on the Co-Cr alloys. In this report, we investigated different ways to analyze the density and binding strength of the apatite layer formed.

We reported that surface hydrophilicity and wettability could be increased after alkali NaOH treatment as confirmed by contact angle analysis, similar enhancement was observed even with only 11-AUPA treatment. However, synergistic effect was observed when NaOH treatment was followed by 11-AUPA treatment. SEM morphology further supports this effect when density of apatite layer formed was improved after both NaOH and 11-AUPA treatment. The results of FT/IR are evidence that NaOH treatment has changed the surface property and there is also increase in intensity for existing -OH groups. In addition, the scotch tape test shows that the apatite layer formed has improved the shear strength or binding to the alloy substrate. The
similar conclusion was drawn with ICP analysis as it shows increased concentration of -OH groups on the surface after NaOH treatment, which is one of the main reasons for improved shear binding strength.

References


[20] Shukla A K, Balasubramaniam R. Effect of surface treatment on electrochemical behavior


[34] Helmy R, Fadeev A Y. Self-assembled monolayers supported on TiO$_2$: comparison of C$_{18}$H$_{37}$SiX$_3$ (X= H, Cl, OCH$_3$), C$_{18}$H$_{37}$Si(CH$_3$)$_2$Cl, and C$_{18}$H$_{37}$PO(OH)$_2$. Langmuir, 2002, 18(23): 8924-8928.


Figure 4-1. Process of the zinc-complex substitution technique.
Figure 4-2. Process of the zinc mixed solution.

50ml of a 4 mol L\(^{-1}\) NH\(_3\)Cl solution + 25ml of a 0.4 mol L\(^{-1}\) ZnCl\(_2\) solution

Adjust the PH of above solution to 6.9 by 30 vol% NH\(_4\)OH solution

Add ultrapure water to the above mixed solution with a PH of 6.9 until 100ml
Figure 4-3. Static contact angle (º) toward distilled water on the surface of Co-Cr specimen under different states.
Figure 4-4. FT/IR spectra of Co-Cr alloy before and after NaOH pretreatment.
Table 4-1. The concentration of zinc ions (ppb) measured by ICP.

(BC: Bare Co-Cr, NC: Co-Cr after NaOH pretreatment)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentration of zinc ions (ppb)</th>
<th>Average of concentration of zinc ions (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC</td>
<td>0.368</td>
<td>0.345</td>
</tr>
<tr>
<td>NC</td>
<td>1.517</td>
<td>1.726</td>
</tr>
</tbody>
</table>
Figure 4-5. XRD patterns of Co-Cr alloys after soaking in SBF for 4 and 7 days.
Figure 4-6. SEM photographs of Co-Cr alloys with and without NaOH pretreatment after soaking in SBF for 4 days and 7 days.
Figure 4-7. SEM photographs of Co-Cr alloys after soaking in SBF for 7 days before and after tape test.
Chapter 5

THE STUDY OF 11-AUPA SELF-ASSEMBLED MONOLAYERS (SAMS) IN INDUCING APATITE FORMATION ON ALUMINA CERAMIC

1. Introduction

Alumina, also known as aluminium oxide (Al$_2$O$_3$), is the only one thermodynamically stable solid oxide form of aluminium [1, 2]. Its abrasion resistance, strength and chemical inertness have made it to be recognized as a ceramic for dental and bone implants [1]. Alumina bioceramic has been developed as an alternative to metal alloys due to their high hardness, low friction coefficient and excellent corrosion resistance [3]. Alumina offers a very low wear rate which makes it a very useful biomaterial [2]. The surface energy and surface smoothness of this ceramic are the reason for its excellent wear and friction behavior [2]. In past, the biocompatibility of alumina has been tested by many researchers [4, 5]. Their evaluation results proved that Alumina ceramic has bioinertness. There have been rare reports of connecting alumina directly with bone owing to its bioinert property. In fact, for the cases of bioactive glasses and glass ceramics the interface with bone is due to the existence of intermediate apatite layer [6-9]. A biologically active bone-like apatite layer necessarily required to form on the surface of implant within the body [10]. Alumina as prostheses has already been used much earlier to bioactive glasses and glass-ceramics. P. Li et al [4] have checked the alumina, silica and titania gel ability to induce the apatite layer using a simulated body fluid (SBF) solution. There results showed that apatite induction occurred with silica gel and titania gel because of their plentiful -OH groups. However, alumina barely interacted with SBF to form calcium phosphate, indicting less affinity of AlOH for calcium and phosphate. Therefore, it was the indication that not all kind of -OH groups could induce apatite.
As we have already researched that SAMs functionalized with 11-AUPA can be formed on the surfaces of Co-Cr alloys and by following $\gamma$-PGA mixed solution immersion, apatite layers could be induced in SBF containing a sufficient amount of Ca$^{2+}$[11]. And it was also reported that self-assembled monolayers (SAMs) can be successfully constructed on the surfaces of alumina ceramic [12-14]. So we try to use this same method to induce apatite in the SBF solution.

In this chapter, we will use 11-aminoundecylphosphonic acid ((11-AUPA):NH$_2$ (CH$_2$)$_{11}$PO$_3$H$_2$·HBr) to prepare the SAMs with the subsequent immobilization with $\gamma$-PGA. The apatite-forming properties will be researched by soaking these samples into SBF solutions for different periods up to 7 days. And we also studied the influence of different concentration of CaCl$_2$ solution on apatite precipitation in a simulated body fluid.

2. Experimental

2.1 Materials

The 11-aminoundecylphosphonic acid purchased from Dojindo Laboratories Co., Ltd. (Kumamoto, Japan), $\gamma$-PGA with a molecular weight in the range of 800 to 1200 kDa purchased from Meiji Seika Kaisha, Ltd. (Tokyo, Japan), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and N-Hydroxysuccinimide (HOSu) purchased from Wako Pure Chemical Industries (Osaka, Japan)were used in our study. Chemical surface modification method was carried on the alumina ceramic purchased from Nihon AS ONE Corp (Osaka, Japan), which has a chemical composition of 99.5%, density of 3.9 g/cm$^3$ and bending strength of 250 MPa. Sodium dodecyl sulfate (SDS) solution, ethanol, acetone were all purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other reagents used in preparing the simulated body fluid were all purchased from
Nacalai Tesque Inc. (Kyoto, Japan). Ultrapure water with a resistivity of 18 MΩ·cm was prepared using a water purification system (Direct-Q, Millipore Co., Billerica, MA, USA).

2.2 Formation of 11-AUPA self-assembled monolayers and γ-PGA monolayers

The alumina ceramic was cut into 10 mm×10 mm×1.0 mm specimens and polished (Buehler AutoMet 2 polishing machine) with #500, 1000 and 1500 waterproof silicon carbide papers. Then the polished specimens were cleaned by ultrasonic vibration in the following order of 0.5% SDS aqueous solution, ethanol, acetone and ultrapure water for every 20 min. Then the samples were immersed in 5ml of 1 M 11-AUPA ethanol solution at 36.5°C for 2 days with a following mildly rinse in ethanol, ultrasonic irradiation in ultrapure water for 10 min and dried in the air. Then all the samples modified by immersion in 11-AUPA were soaked in an aqueous mixed solution containing 2.0% (Mass content) γ-PGA, 3.1% EDC·HCl and 1.8% HOSu under stirring for 1 day at room temperature.

2.3 SBF soaking treatment

In order to explore the bioactivity inducing ability of alumina ceramic by chemical surface modification, the samples were soaked in 30 ml of SBF (Na⁺:142.0, K⁺:5.0, Mg²⁺: 2.5, Cl⁻: 147.8, HCO₃⁻: 4.2, HPO₄²⁻: 1.0, and SO₄²⁻: 0.5 mM, PH7.40) at 36.5°C for various periods of up to 7 days after the immersion dispose in 0.1 M, 0.5 M and 1 M CaCl₂ solution. After SBF soaking treatment, the samples were immersed in ultrapure water for 1 day and then dried at room temperature.

2.4 Surface characterization

The untreated samples after cleaning and modified by 11-AUPA SAMs and γ-PGA solution were characterized by contact angle measurement (DMe-200, Kyowa Interface Science
Co., Ltd., Saitama, Japan). The static contact angles toward distilled water were measured, which were the mean values of at least three different spots on each of the three different specimens.

2.5 Surface structural features

The surface structure changes of the samples before and after SBF soaking treatment were characterized by thin-film X-ray diffraction (TF-XRD; MXP3V, Mac Science Ltd., Yokohama, Japan) and scanning electron microscopy (SEM; S-3500N, Hitachi Co., Tokyo, Japan). For TF-XRD, the angle of the incident beam was fixed at 1° against the surface of the samples and the measurements were performed using a step scanning mode with steps at 0.02° steps and 1s.

2.6 Scotch Tape test

In order to evaluate the adhesion ability between the apatite and the substrates, Scotch Tape Test was used in this experiment. The main process was to cover the half surface of the samples by the tape and then peeled it off gently. According to the surface structure changes of the samples before and after scotch tape test by SEM analysis, we can judge the binding force of the apatite to the substrates.

3. Results and discussion

3.1 Contact angle analysis

Figure 5-1 shows the contact angle images toward distilled water for alumina ceramic without and with modification of 11-AUPA immersion and following mixed $\gamma$-PGA solution soaking. In comparison with the raw $\text{Al}_2\text{O}_3$ substrate, the contact angle values decrease obviously from 46.8° to 27.4° after the treatments with 11-AUPA immersion and following mixed $\gamma$-PGA
solution soaking, which indicates the surface wetting ability have improved. The wetting characteristics were due to some hydrophilic groups immobilized on to the surface after modification with both treatments. According to the same phenomenon we discussed in chapter 2 and 3, this change was due to the successful immobilization of $\gamma$-PGA, which is hydrophilic.

### 3.2 XRD analysis

The TF-XRD patterns of the $\text{Al}_2\text{O}_3$ ceramic were recorded following their sequential treatment with 11-AUPA, $\gamma$-PGA and different concentrations of CaCl$_2$ in SBF for 1, 4 and 7 days respectively, and the results are shown in Figure 5-2. In the cases of the specimen with 0.1 M CaCl$_2$ treatment before soaking in SBF as shown in Figure 5-2-1, although we extended the soaking time from 1 day to 7 days, there were no new peaks observed except the peaks those assigned to the raw $\text{Al}_2\text{O}_3$ material. When we increased the concentration of CaCl$_2$ solution from 0.1 M to 0.5 M shown in Figure 5-2-2, we can clearly observe the new broad peaks appeared at about 26º and 32º after soaking in SBF for 7 days, which indicates the successful apatite formation. On the other hand, with the increase in concentration of CaCl$_2$ solution to 1 M shown in Figure 5-2-3, apatite formation can be achieved even after soaking in SBF for 4 days. This phenomenon indicates that the nucleation of apatite need a relative high concentration of Ca$^{2+}$ environment and the nucleation speed could be accelerated by increasing CaCl$_2$ concentration. At the same time, with extending the soaking time in SBF in the cases of 1 M CaCl$_2$ solution treatment the intensity increase of apatite accompanied by the decreasing intensity of $\text{Al}_2\text{O}_3$ material which indicates more amount of apatite formation on the substrates.

### 3.3 SEM analysis

-100-
SEM images of the Al₂O₃ material before and after their sequential treatment with 11-AUPA and γ-PGA are shown in Figure 5-3. No significant differences were observed after the chemical surface modification.

Figure 5-4 shows the SEM images of the Al₂O₃ ceramic when treated with different concentration of CaCl₂ solutions after soaking SBF for 1, 4 and 7 days. In accordance with the results of TF-XRD patterns (Fig. 5-2), there are hardly changes before and after soaking in SBF for 1, 4 and 7 days when the samples treated with 0.1 M concentration of CaCl₂ solutions (Fig. 5-4-1).

In the cases of increasing the concentration of CaCl₂ solution to 0.5 M, some spherical particles were formed on the surface of the substrates and the amount of these particles increased obviously to form an apatite layer after extending the soaking time in SBF for 7 days. At the same time, more apatite particles grew upon the formed layer. As the amount of apatite particles was large in the case of soaking in SBF for 7 days, so we can get the apatite peaks from the XRD patterns, however we can’t get new peaks in the cases of specimen soaking in SBF for 4 days due to the limited amount of apatite.

As we increased the concentration of CaCl₂ solution to 1 M, the nucleation speed increased due to high concentration Ca²⁺, we can observe some apatite particles even after soaking in SBF for 1 day, as shown in Figure 5-4-3. With extending the soaking time in SBF for 4 days, more apatite particles appeared and almost formed the apatite layer with a whole coverage of the surface. In the case of sample soaking in SBF for 7 days, this effect works more as a result of more big and dense apatite particles formed on the apatite layer in comparison with the samples treated with 0.1 M and 0.5 M CaCl₂ solution. Figure 5-5 shows the SEM images of the section part of the samples treated with 1 M CaCl₂ solution after soaking in SBF for 7 days. We can clearly see that the thickness of apatite layer was about 3-4 μm and the big apatite particles grew upon it.
3.4 Tape test

A very simple way to check the binding strength between the apatite layer and the Al$_2$O$_3$ ceramic is the scotch tape test, namely, to check the structure changes of the surface before and after tape adhesion, as shown in Figure 5-6. After tape test, the Al$_2$O$_3$ ceramic surface transfer into a rough etched surface from a dense apatite layer surface. This effect was obvious under high magnification SEM photograph showed in the red area. The original area covered by little dark apatite layer disappeared becoming into heavy dark which indicates the apatite layer was peeled off after tape test. So the tape test result obtained indicates the binding strength between the apatite layer and the Al$_2$O$_3$ ceramic surface is not strong enough and need more work to improve its binding force.

4. Conclusions

SAMs functionalized with 11-AUPA were formed on the surfaces of Al$_2$O$_3$ ceramic in aqueous solution. γ-PGA was then immobilized on the SAMs via a covalent cross-linking reaction. Apatite layers subsequently formed on the surfaces of the modified alloys when they were submerged in SBF containing a sufficient amount of Ca$^{2+}$. The concentration of Ca$^{2+}$ plays a very important role in inducing and accelerating apatite forming speed.

The results of this study therefore represent a novel surface modification method that can be used to increase the bioactivity of Al$_2$O$_3$ ceramic.
References


Tables and Figures

Figure 5-1. Contact angle images for Al$_2$O$_3$ before and after modification of 11-AUPA immersion and following mixed γ-PGA solution soaking.
Figure 5-2. TF-XRD patterns of the surfaces of Al₂O₃ ceramic treated with various concentrations of CaCl₂ before and after soaking in SBF (a: Bare Al₂O₃, b: Treated Al₂O₃ + SBF 1d, c: Treated Al₂O₃ + SBF 4d, d: Treated Al₂O₃ + SBF 7d).
Figure 5-2. (Continued)
Figure 5-3. SEM images of the surfaces of Al$_2$O$_3$ ceramic with and without 11-AUPA and subsequent γ-PGA treatments.
Figure 5-4. SEM images of Al₂O₃ ceramic treated with different concentration of CaCl₂ solutions after soaking in SBF for 1, 4 and 7 days.
5-4-3  1 M CaCl$_2$

**Figure 5-4.** (Continued)
Figure 5-5. SEM images of Al₂O₃ ceramic section treated with 1 M CaCl₂ solution after soaking in SBF for 7 days.
Figure 5-6. SEM images of Al₂O₃ ceramic treated with 1 M CaCl₂ solution after soaking in SBF for 7 days before and after tape test.
Chapter 6

GENERAL CONCLUSION

Possibility of using Cobalt-chromium alloy (Co-Cr) and alumina (Al₂O₃) as bioactive material was achieved successfully via various surface modifications and by the formation of intermediate SAM layers. The main idea was to introduce the apatite layer forming ability on the surfaces of the Co-Cr and alumina for their application as implant biomaterials. Efforts were directed to further increase the density of apatite layer and improve the shear binding strength between the apatite layers formed and implant surface.

In the first chapter of the thesis, I did some literature survey on the different kind of biomaterials used during different generations. There I learned about the limitations associated with the use of different biomaterials for implant applications, which gave me an idea and possible area of research. Therefore, Cobalt-chromium (Co-Cr) alloy and Alumina (Al₂O₃) were taken for the current study as both are bioinert materials i.e they cannot induce apatite in their native state. However, the property such as high strength, ductility, malleability, and temperature endurance, pitting resistance, crevice corrosion resistance and wear resistance makes Co-Cr an important biomaterial. Similarly, alumina shows excellent wear rates, excellent corrosion resistance, good biocompatibility and high strength.

Second chapter deals with my beginning of experimental work regarding proposal of a novel surface modification method that can be used to increase the bioactivity of bioinert materials. In this context, surface modification of Co-Cr alloy was demonstrated. SAMs functionalized with 10-CDPA were formed on the surfaces of Co-Cr alloys by dipping method. The results of this study demonstrated that the surface modification method by only 10-CDPA could lead to the improvement of bioactivity behavior. In this context, the apatite layers were
induced by the subsequent immersion in SBF with CaCl$_2$ treatment containing a sufficient amount of Ca$^{2+}$. Here I showed that the heat treatment can improve the binding ability of the apatite layer with the substrate.

In the chapter three, my work was by using 11-AUPA phosphonic acid as SAM layer. A covalent cross-link thus formed with the immobilization of γ-PGA on the SAM layer. Apatite layers subsequently formed on the surfaces of the modified alloys when they were submerged in SBF containing a sufficient amount of Ca$^{2+}$. Also, results show that HOSu plays an important role in final apatite induction.

Chapter four was mainly focused on increasing the density of the apatite layer on the Co-Cr surface and also its binding strength with the surface. An approach utilized for this was the use of sodium hydroxide (NaOH), which increased the –OH (hydroxyl) groups on the surface. This increase in –OH further improved the coverage and formation of SAM layer using 11-AUPA. Results showed that surface hydrophilicity and wettability was increased after treatments as confirmed by contact angle analysis. In addition results showed that the apatite layer formed has improved shear strength or binding to the alloy substrate mainly because of the increased concentration of –OH groups on the surface after NaOH treatment.

In the final chapter of my thesis I used alumina for introducing the bioactivity on its surface. Similar methods used for introducing bioactivity on Co-Cr as in chapter second were implemented for the alumina surface also. The concentration of Ca$^{2+}$ plays a very important role in inducing and accelerating apatite forming speed. But the binding strength is not strong enough and need more work to improve it.

The results of this study therefore represent a novel surface modification method that can be used to increase the bioactivity of bioinert materials.
ACHIEVEMENTS

List of Publication


List of Conference Presentation


3. C. Liu, T. Miyazaki, Y. Shirosaki, “Apatite precipitation on cobalt-chromium (Co-Cr) alloys modified with γ-polyglutamic acid in a simulated body fluid”, *14th Asian..."


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