Effects of pH, Potential, and Deposition Time on the Durability of Collagen Electrodeposited to Titanium

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Collagen is expected to work as a bonding agent of soft and hard tissues to solid materials. In this study, the electrodeposition of collagen to a titanium (Ti) surface under various conditions, i.e., the pH of the collagen solution, potential, and electrodeposition time, was performed to understand the optimal electrodeposition conditions for the immobilization of collagen to Ti. The effects of these conditions on the thickness and residual ratio of the collagen layer after shaking in water were evaluated by ellipsometry, scanning probe microscopy, and X-ray photoelectron spectroscopy. Collagen molecules were attracted to Ti cathode and immobilized with high durability by combining electrodeposition conditions, pH 5, alternating potential between −1 V and +1 V vs. SCE with 1 Hz, and 1800 s. The surface of this electrodeposited collagen layer was smooth and uniform maintaining the collagen fibril and natural structure. On the other hand, the collagen layer immobilized by immersion technique in a collagen solution, was rough and irregular. Electrodeposition with alternating potential at pH 5 for 1800 s is a much more appropriate technique to immobilize collagen to Ti than the conventional immersion technique.

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1. Introduction

Titanium (Ti) and Ti alloys are widely used as medical implant devices due to their good tissue compatibility. In particular, dental implants, orthodontic implant anchorages, percutaneous devices, and the screws of external fixators penetrate the human body from inside to outside. Unless living tissue contact these devices, a crevice between the tissue and device is generated, bacteria invade through the crevice, and infection and/or loosening occurs. Adhesion of soft tissue to a metal surface is broken by external forces and bacterial toxins because of weak adhesion and the lack of an adhesive structure. To improve the tissue compatibility or cell adhesion of a metal surface, a surface treatment is necessary. Most surface treatments studied for the last two decades aim at the improvement of hard tissue compatibility and the acceleration of bone growth on Ti; however, the number of studies on the improvement of the soft tissue compatibility of Ti is much smaller than that of studies on hard tissue compatibility. The immobilization of collagen and laminin is effective to improve the soft tissue compatibility of Ti. However, studies on the immobilization of bioactive proteins and peptide, e.g., collagen, RGD, and BMP, are conducted for the promotion of osseointegration or hard tissue compatibility. In addition, the immobilization of proteins is performed by immersion technique in the above studies, and proteins are easily exfoliated due to low reliability of the adhesive strength. Furthermore, the thickness of the immobilized layer is not controlled and is not uniform. Segregation in the thickness of the immobilized layer weakens the bonding strength of proteins and depletes tissue integration to the metal surface. Therefore, immobilization techniques to perform strong adsorption with high uniformity and durability are required.

Various functional molecules and biomolecules are immobilized by electrodeposition on a Ti surface. For example, immobilized both-terminals-terminated poly(ethylene glycol) (NH₂-PEG-NH₂), is uniformly immobilized with electrodeposition, and the durability of the immobilized layer is large because of the strong interaction of the terminal (NH₄⁺) and hydroxyl groups (O⁻) of the surface oxide on the Ti. The immobilized PEG layer inhibits the adsorption of albumin and the adhesion of platelet and bacteria. On the other hand, the electrodeposition layer of collagen on Ti with uniformity and durability is expected to accelerate cell adhesion. The durability of the immobilized collagen layer is unknown, while immobilization at various pH values and both anodic and cathodic potentials has been performed.

Therefore, the optimal conditions, i.e., the pH, potential, and time for the immobilization of collagen to Ti with large durability, were investigated to obtain hard and soft tissue compatibility.

2. Experimental

2.1 Preparation of a Ti substrate

Commercially pure Ti disks (8 mm in diameter and 1.5 mm in thickness) with grade 2 (Rare Metallic Co., Japan) were polished with SiC papers, a 9 μm diamond suspension, and, finally, a 0.04 μm colloidal-silica suspension to obtain a flat, relatively clean, and shiny surface. The Ti disks were cleaned from macroscopic contamination by ultrasonic in acetone for 900 s and dried with a stream of nitrogen (99.9%). To obtain a stable passive oxide film on the Ti, the disks were placed in a desiccator for more than 24 h.
2.2 Immobilization with electrodeposition

The Ti disk was fixed in a polytetrafluoroethylene holder that was insulated from the electrolyte except for an opening made for electrodeposition (6.0 mm). Therefore, the area exposed for electrodeposition was 28.3 mm².

Type I collagen from pig (Research Institute for the Functional Peptides, Japan) was dissolved in a 0.13-M NaCl solution with a concentration of 10 mg mL⁻¹. The pH of the solution was adjusted to 3, 5, 6, and 9 by a NaOH solution. The resultant solution was used as the electrolyte for electrodeposition.

Electrodeposition was performed at 298 K. The open circuit potential, OCP, of Ti vs. the saturated calomel electrode, SCE, was measured as ca. -0.2 V. Thereafter, a direct current (DC) by a cathodic potential or a direct anodic potential was charged at -1 V SCE or +1 V SCE (abbreviated as DC₋₁V or DC₊₁V), and the potential was maintained for 30 s and 1800 s. In addition, an alternating current (AC) was charged with a cyclic potential on a sine curve between -1 and +1 V SCE with a frequency of 1 Hz. The currents as these potentials were charged were monitored as shown in Fig. 1. The abbreviation of each specimen was given in Table 1.

With a charging potential, collagen molecules were attracted to the Ti electrode and immobilized on it. After electrodeposition, specimens were rinsed in deionized water and dried with a stream of nitrogen gas (99.9%).

The electrodeposition system in this study is illustrated in Fig. 2, and it is the same as that used in a previous study.12)

The flowchart of experiments and conditions are shown in Fig. 3. Electrodeposition was first conducted by DC₋₁V, 30 s in solutions with pH 3, 5, 6, and 9. The electrodeposited layer of collagen was characterized by ellipsometry and scanning probe microscopy (SPM). On the other hand, electrodeposition was performed in a constant pH of 3 by various potentials for 30 s and 1800 s. The electrodeposited layer was characterized by ellipsometry, SPM, and X-ray photoelectron spectroscopy (XPS). The durability of the immobilized layer was evaluated as follows. Specimens were in shaken 2-mL distilled water (Millipore) at ambient temperature for 72 h using a water bath shaker (150 min⁻¹, Personal-11, TAITEC Co., Japan). After being shaken, each specimen was rinsed in distilled water and dried with a stream of nitrogen (99.9%). The thickness of the residual layer was determined by ellipsometry. The residual ratio of immobilized collagen after shaking was calculated using the thicknesses before and after shaking. This residual ratio was used as a durability index.

According to the above results, an optimal condition for the electrodeposition of collagen was determined, and the electrodeposition by the condition was performed. The result was compared with that of immersion by ellipsometry and SPM.

2.3 Immobilization with immersion technique

The collagen was originally dissolved in a HCl solution with a concentration of 0.01 g mL⁻¹ when it was delivered. The pH of the solution was adjusted as pH 3 with HCl. The Ti disks were immersed and collagen was adsorbed on the Ti surface in the solution at 4°C for 24 h. After adsorption, the disks were removed from the solution remaining layer on it and dried in air. This process followed conventional collagen immobilization technique.

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Table 1 Conditions of electric potential and electrodeposition time and their abbreviations.

<table>
<thead>
<tr>
<th>Condition</th>
<th>30 s</th>
<th>1800 s</th>
</tr>
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<tbody>
<tr>
<td>DC with -1 V SCE</td>
<td>DC₋₁V₃₀s</td>
<td>DC₋₁V₁₈₀₀ₙ</td>
</tr>
<tr>
<td>AC</td>
<td>AC₃₀ₙ</td>
<td>AC₁₈₀₀ₙ</td>
</tr>
<tr>
<td>DC with +1 V SCE</td>
<td>—</td>
<td>DC₊₁V₁₈₀₀ₙ</td>
</tr>
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Fig. 1 Responded current to charged electric potentials: (a) DC₋₁V, (b) AC, (c) DC₊₁V.

Fig. 2 Schematic illustration of electrodeposition in the case of negative potential charged.
2.4 Ellipsometry
The apparent thickness of the immobilized collagen layer on Ti was determined by ellipsometry (DVA-36Ls, Mizojiri Optical Co., Ltd.) in air. The thickness determined in air was possibly underestimated compared to that in solutions. The light source was a He-Ne laser with a wavelength of 632.8 nm, and the incident angle to the Ti surface was 70°. The thickness was calculated by optical constants: the refractive index and absorption coefficient of Ti oxide with the Ti substrate were 2.209 and 3.079,20,21) and those of the Ti substrate were 2.22 and 2.99,22) respectively.

2.5 SPM
The surfaces of specimens were imaged by the dynamic force mode with SPM (SPM9600, Shimazu, Japan) using silicon cantilevers (NCHR-20, Nano World AG, Switzerland) with a blade thickness of 4 μm, a width of 30 μm, and a resonance frequency of 320 kHz in air.

2.6 XPS
The immobilization manner of collagen and chemical state of the surface oxide film on Ti were characterized using XPS (SSI-SSX100). The take-off angle for photoelectron detection was 35° to the surface of specimens. All binding energies given in this paper are relative to the Fermi level,

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Fig. 3 Flowchart of experiments in this study.
and all spectra were excited with the monochromatized Al Kα line (1486.61 eV). The spectrometer was calibrated against Au 4f7/2 (binding energy, 84.07 eV) and Au 4f5/2 (87.74 eV) of pure gold and Cu 2p3/2 (932.53 eV), Cu 2p1/2 (952.35 eV), and Cu Auger L3M45M45 line (kinetic energy, 918.65 eV) of pure copper. The energy values were based on published data.23) The binding energy on the XPS spectra was corrected according to a peak binding energy of C 1s electrons (originating from hydrocarbon C-C and C-H; Fig. 4) thickness and residual ratio of the immobilized collagen layer by electrodeposition at various pH values before and after shaking. The residual ratio decreased with the electrodeposition time in DC specimens (AC−1V,1800s, +1V,1800s), while it increased in AC specimens (AC−1V,30s, AC1800s, AC−1V,1800s). The residual ratio decreased with the electrodeposition time in DC specimens (DC−1V,1800s, DC−1V,30s, DC+1V,1800s), while it increased in AC specimens (AC−1V,30s, AC+1V,1800s). AC1800s had the highest durability in the five conditions.

From the viewpoint of the durability and thickness of the collagen layer, the best pH and the best electrical potential were pH 5 and AC1800s, respectively, from Figs. 4 and 5 results. The thickness and residual ratio of electrodeposited collagen layer at the best condition were compared with those of collagen immersion technique (Fig. 6). The thickness of collagen layer adsorbed by immersion technique was larger than those in electrodeposition specimens, while the residual ratio and uniformity were smaller. The residual ratio of the specimens with AC1800s at pH 5 were the highest among all conditions.

Figure 5 shows the thickness of the immobilized collagen layer on specimens prepared with various potentials and times at pH 3 before and after shaking. This figure represents the effect of the potential and time on the thickness and durability of immobilized collagen. The thickness was the largest in DC−1V,1800s. The layers by AC−1V,30s and AC+1V,1800s were thicker than those by DC−1V,30s and DC+1V,1800s. The residual ratio decreased with the electrodeposition time in DC specimens (DC−1V,30s and DC−1V,1800s), while it increased in AC specimens (AC30s and AC+1V,1800s). AC1800s had the highest durability in the five conditions.
3.2 Surface topography of collagen layer onto Ti

Figure 7(a) shows an SPM image of untreated Ti, and the surface is smooth without projection originating from electrodeposited collagen. One projection appeared on the surface is a particle from the polishing reagent. Figures 7(b), 7(c), 7(d), and 7(e) show Ti-electrodeposited collagen at pH 3, 5, 6, and 9, respectively, after shaking (1000 nm x 1000 nm x 10 nm in the scale of the picture), and high magnification image of (c) (f) (200 nm x 200 nm x 5 nm in the scale of the picture).

Fig. 7 SPM images of untreated Ti (a) and the collagen layer immobilized on Ti with electrodeposition at pH 3 (b), pH 5 (c), pH 6 (d), and pH 9 (e) after shaking (1000 nm x 1000 nm x 10 nm in the scale of the picture), and high magnification image of (c) (f) (200 nm x 200 nm x 5 nm in the scale of the picture).

Fig. 8 SPM images of the collagen layer immobilized on Ti with (a) DC_{−1V,1800s}, (b) AC_{1800s}, and (c) DC_{+1V,1800s} (1000 nm x 1000 nm x 10 nm in the scale of the picture).

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Figure 7(a) shows an SPM image of untreated Ti, and the surface is smooth without projection originating from electrodeposited collagen. One projection appeared on the surface is a particle from the polishing reagent. Figures 7(b), 7(c), 7(d), and 7(e) show Ti-electrodeposited collagen at pH 3, 5, 6, and 9, respectively, after shaking in water (1000 nm x 1000 nm x 10 nm). At all pH values, granular and small projections were observed in addition to large projections from the polishing reagent. On the other hand, fibrous structures were observed on the substrate only at pH 5. Figure 7(f) shows a magnified image of Fig. 7(c) (200 nm x 200 nm x 5 nm). Collagen fibrils on the surface of Fig. 7(c) observed clearly.

Figure 8 shows the SPM images of Ti-electrodeposited collagen by three kinds of electric potential conditions after shaking: DC_{−1V,1800s}, AC_{1800s}, and DC_{+1V,1800s}. In the DC_{+1V,1800s} specimen (Fig. 8(c)), no collagen fibril was observed, but the surface was rough. The collagen layer on the DC_{−1V,1800s} specimen (Fig. 8(a)) was much thinner than
that on the AC_{1800s} specimen (Fig. 8(b)). In particular, collagen fibrils were aggregated on the AC_{1800s} specimen.

Figure 9(a) shows the image of Ti-electrodeposited collagen with AC_{1800s} at pH 5, whose condition was the best combination of pH, potential, and time, according to the above results. Collagen existed as a fibrillar structure, and fibrils made up the smooth network maintaining natural structure. Figure 9(b) is a cross section of Fig. 9(a). The thickness of the collagen fibril was about 4 to 6 nm. Figure 9(c) shows Ti immobilized collagen by immersion technique, which is a conventional technique for the immobilization of collagen. The surface was rough and irregular.

3.3 State analysis of the immobilized collagen layer

XPS was performed for untreated Ti and Ti electrodeposited collagen under DC_{−1V,1800s}, AC_{1800s}, and DC_{+1V,1800s} conditions. C, N, O, and Ti were detected using XPS. Figures 10(a), 10(b), 10(c), and 10(d) show the XPS spectra of Ti 2p, N 1s, C 1s, and O 1s electron energy regions, respectively, and untreated Ti and decomposition of the peaks to component peaks.

The Ti 2P_{3/2} peak was decomposed to four peaks originating from the metal state as Ti^0 and the oxide states as Ti^{2+}, Ti^{3+}, and Ti^{4+}, as shown in Fig. 10(a). The proportions of the integrated intensities of Ti^0, Ti^{2+}, Ti^{3+}, and Ti^{4+} among the total intensity of the Ti 2P_{3/2} peak are summarized in Table 2. When the oxide film is grown, the
sum of the integrated intensities of Ti\(^{2+}\), Ti\(^{3+}\), and Ti\(^{4+}\) increases, and that of Ti\(^0\) decreases. Therefore, the thickness of the surface oxide film was the largest in this order: DC\(_{+1800}\), AC\(_{1800}\), untreated Ti, and DC\(_{-1800}\). The thicknesses of the surface oxide film of untreated Ti and DC\(_{-1800}\) are almost equal as shown in Table 3. DC\(_{+1800}\) was the largest (about 2.0 nm thicker than untreated sample) and AC\(_{1800}\) was the second largest (about 1.0 nm thicker than untreated Ti). DC\(_{+1800}\) works as anodic polarization that induces the growth of the surface oxide film. On the other hand, DC\(_{-1800}\) works as cathodic polarization, but the thickness of the film do not change because the cathodic current is mainly used for the hydrogen evolution. In the case of AC\(_{1800}\), anodic and cathodic polarization is repeated. The surface oxide film slightly grows when anodic potential was charged.

The N 1s peak consists of only one peak originating from peptide bonding ([C(O)NH] peak) (Fig. 10(b)). The integrated intensity of the N 1s peak is governed by the immobilized amount of collagen. The integrated intensities of N 1s and Ti 2p peaks increase and decrease, respectively, when the amount of collagen increases. The ratio of the integrated intensity of the Ti 2p\(_{3/2}\) peak to that of N 1s, \([\text{Ti} 2p_{3/2}] / [\text{N} 1s]\), was calculated as listed in Table 4. As immobilized collagen immobilized larger, the ratio become smaller. In this study, the ratio was the smallest in this order: AC\(_{1800}\), DC\(_{-1800}\), DC\(_{+1800}\), and untreated Ti.

The C 1s peak was decomposed to four component peaks originating from C-C and C-H, C-O and C-N, C(O)NH, and C=O, as shown in Fig. 10(c). The proportions of the integrated intensities of C-C and C-H, C-O and C-N, C(O)NH, and C=O among the total intensity of the C 1s peak are summarized in Table 5. Most of the C-C and C-H bonds originated from contamination. C=O exists in peptide bonding, the lateral chain, and the terminal; C-N exists in peptide bonding, the lateral chain, and the terminal of collagen; and C-O exists in the lateral chain and the terminal. The C(O)NH peak originating from peptide bonding was clearly identified in DC\(_{-1800}\), AC\(_{1800}\), and DC\(_{+1800}\) specimens, and the peak was the largest in the AC\(_{1800}\) specimen.

The O 1s peak is decomposed to peaks originating from O\(^2-\), OH\(^-\), and C=O, and H\(_2\)O. The proportions of the integrated intensities of O\(^2-\), OH\(^-\) and C=O, and H\(_2\)O among the total intensity of the O 1s peak are summarized in Table 6. The proportion of OH\(^-\) and/or C=O was the largest in this order: DC\(_{-1800}\), DC\(_{+1800}\), AC\(_{1800}\), and untreated Ti, as shown in Fig. 10(d). In the DC\(_{-1800}\) specimen, the surface oxide film was reduced, OH\(^-\) increased by the cathodic potential, and the thickness decreased.

4. Discussion

4.1 The effect of pH on electrodeposition
Collagen fibrils become unstable under a high pH condition. Collagen denaturation is a phenomenon in which the collagen triple helix is loosened and changes to gelatin. The gelatin is mechanically weak, easily decomposed, and absorbed in the living body because it easily dissolves in an aqueous solution. Therefore, pH adjustment was required for treatments using collagen.

The average of the residual ratio of specimens electrodeposited at pH 9 was about 97.7%, and it was the largest, as shown in Fig. 4. The exfoliation of collagen rarely occurred...
at pH 9. This is possibly caused by the denaturation of collagen. Both large thickness due to swelling and extremely small thickness due to exfoliation were simultaneously occurred. This induced the increase of apparent thickness and the residual ratio in pH 9.

On the other hand, the thickness of the collagen layer on the specimen prepared at pH 5 was the second rank remaining collagen fibril and its natural structure (Figs. 4 and 7). Monomeric collagen self-assembles to form native-type fibrils in vitro (reconstitution) under physiological conditions. The reconstituted collagen fibrils exhibit improved mechanical properties and biological stability over those of the monomeric form. The apparent thickness shown in Fig. 4 is governed by the density and thickness of collagen fibrils. The isoelectric point of collagen is about 9, and collagen is mainly positively charged, while it has positive and negative charges in the lateral chains and terminals at pH 5 and 6. Collagen was more positively charged at pH 5 than pH 6. Therefore, collagen molecules were more attracted to the Ti cathode at pH 5.

Moreover, because of electrode reaction, localized pH at the cathode is increased due to the generation of OH. To prevent the denaturation of collagen, pH 5 is a better condition than pH 6.

4.2 The effect of electric potential and time on electrodeposition

In the DC+1V,1800s specimen, no collagen fibril was observed, but the surface was rough (Fig. 8), while the apparent thickness was the largest, as shown in Fig. 5. In addition, the oxide film was grown in the DC+1V,1800s specimen, as determined with XPS and shown in Fig. 10(a) and Table 3, because anodic polarization makes it possible for the surface oxide film to grow easily. In other words, the largest thickness in DC+1V,1800s, as shown in Fig. 5, is mainly caused by the growth of the surface oxide film on Ti due to anodic polarization. Therefore, the thickness of the electrodeposited collagen layer is unclear. The condition DC+1V,1800s is not suitable for electrodeposition.

With the exception of DC+1V,1800s, the AC1800s specimen had the thickest collagen layer (Fig. 5), and the ratio [Ti 2p3/2]/[N 1s] was also the smallest (Table 4). In other words, the amount of immobilized collagen was the largest in AC1800s because both positively and negatively charged sites of collagen could be attracted to the Ti surface by the alternating current and effectively immobilized. In the previous study, calcium phosphate was successfully electro-deposited to Ti by AC with 1 Hz because the calcium ion is positive and the phosphate ion is negative in the electrolyte. In Fig. 1, current generated by DC−1V and DC+1V immediately increased and decreased just after charging and remained at small constant values. On the contrary in AC, positive and negative currents were repeatedly generated for a long time after charging. In AC, the lateral chains and terminals with radicals (OH−, COO−, NH3+) of electrodeposited collagen fibrils not combined with Ti were attracted to the Ti surface, and collagen fibrils were then immobilized next to each other. Therefore, electrodeposition at AC and longer time of 1800 s are suitable for collagen immobilization.

4.3 Comparison of electrodeposition by optimal condition with the conventional immersion technique

A specimen prepared with AC1800s at pH 5 showed the largest thickness and residual ratio of all the electrodeposited collagen layer remaining fibril structure (Figs. 6 and 9(a)). In pH 5, the collagen fibril has a lot of positive charges that are attracted to a Ti cathode, and Ti surface has positive and negative charges in the pH. In addition, the alternating current remains for a long time after charging, and free radicals of collagen are continuously attracted even after being immobilized. Therefore, the durability increased, and the immobilized layer was smooth and uniform (Fig. 9(a)). On the other hand, the immobilized collagen layer was rough and irregular (Fig. 9(c)). Electrodeposition with an alternating current at pH 5 for 1800 s is a much more appropriate technique than the conventional immersion technique.

5. Conclusions

With the exception of pH 9, collagen fibrils were more attracted to a Ti cathode, and the durability of the immobilized layer was the largest at pH 5 because collagen was more positively charged at pH 5 than pH 6. In pH 9, collagen fibrils were denatured. On the other hand, the alternating potential generated the thickest collagen layer with the fibrous network and the largest durability in water. Therefore, electrodeposition with an alternating current at pH 5 for 1800 s is a much more appropriate technique than the conventional immersion technique.

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