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# Immobilization of epidermal growth factor on titanium and stainless steel surfaces via dopamine treatment

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## ABSTRACT

Titanium and stainless steel were modified with dopamine for the immobilization of biomolecules, epidermal growth factor (EGF). First, the treatment of metal surfaces with a dopamine solution under different pH conditions was investigated. At higher pH, the dopamine solution turned brown and formed precipitates. Treatment of the metals with dopamine at pH 8.5 also resulted in the development of brown color at the surface of the metals. The hydrophobicity of the surfaces increased after treatment with dopamine, independently of pH. X-ray photoelectron spectroscopy revealed the formation of a significant amount of an organic layer on both surfaces at pH 8.5. According to ellipsometry measurements, the organic layer formed at pH 8.5 was about 1000 times as thick as that formed at pH 4.5. The amount of amino groups in the layer formed at pH 8.5 was also higher than that observed in the layer formed at pH 4.5. EGF molecules were immobilized onto the dopamine-treated surfaces via a coupling reaction using carbodiimide. A greater amount of EGF was immobilized on surfaces treated at pH 8.5 compared with pH 4.5. Significantly higher growth of rat fibroblast cells was observed on the two EGF-immobilized surfaces compared with non-immobilized surfaces in the presence of EGF. The present study demonstrated that metals can become bioactive via the surface immobilization of a growth factor and that the effect of the immobilized growth factor on metals was greater than that of soluble growth factor.

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## 1. Introduction

Metals, such as titanium, titanium alloy, and stainless steel implants, are used widely in medicine based on their biocompatibility, nontoxicity, good mechanical properties, and excellent corrosion resistance. Over 70% of implant devices are made of metal [1]. Recently, various surface modifications have been developed to endow biological functionality on metals [1–4]. Surface properties are of prime importance in establishing the response of tissues to biomaterials and provide a set of very powerful signals for cells.

To modify the surface of metals, various organic molecules, from synthetic polymers to proteins, have been developed by many researchers. In addition to surface modifications aimed at preventing biofouling [1,5,6], extracellular matrix, active peptides, or derivatives have been immobilized on metals to promote cell adhesion [7–13].

Moreover, immobilization of proteins on metals was also developed to regulate higher levels of cell function, such as the control of growth or differentiation [14–17]. Various methods, including physical, ionic, and covalent immobilization, have been used for the immobilization of organic molecules on metal surfaces. Among the immobilization methods available, covalent immobilization is considered very useful, because it provides stable immobilization and leads to a biosignaling effect that is maintained for a long period [4,18,19].

Treatment of metal surfaces with silane derivatives has been used widely to achieve covalent immobilization [12,13]. In addition to silane derivatives, Weber et al. synthesized 1-aziglycoses, which react with the hydroxyl groups on the surface of oxidized titanium [20]. The chemicals generated singlet carbenes that were inserted readily into H–O bonds, leading to the glycosidation of titanium. Mikulec and Puleo used p-nitrophenyl chloroformate to immobilize trypsin on the titanium alloy Ti-6Al-4V [21]. Puleo et al. developed a plasma surface modification via the polymerization of allylamine, to enable the immobilization of bioactive molecules on a “bioinert” metal, Ti-6Al-4V [22]. A photoimmobilization method using perfluorophenyl azide groups has also been reported [23]. Recently, Li and coworkers

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develop a new method to treat the surface of titanium via photochemical grafting using alkene-containing compounds attached covalently to the  $\text{TiO}_2$  group, probably via  $\text{Ti}-\text{O}-\text{C}$  bondage [24]. Li et al. reported the development of a “clickable” organic layer via extension of this method [25].

In addition to these methods, biomimetic approaches inspired by mussel adhesive activity have also been devised [26–29]. Because mussel proteins contain 3,4-dihydroxy-L-phenylalanine (DOPA), DOPA has been used by many researchers as a surface treatment to prepare organic layers [30–38]. The similar compound dopamine has also been used [39,40]. Dopamine was attached to polymers and the polymer derivatives were used to achieve surface modification of metals [41–44]. Subsequently, Lee et al. found that the coexistence of a catechol (DOPA) and an amine (Lys), which are rich in mussel's adhesive, was crucial to achieve adhesion to a wide spectrum of materials [45]. This reaction is considered as the formation of a dopamine-melanin layer on the surface [46,47]. These treatments provide not only organic layers but also further immobilization of biological molecules on the surface of various materials [48–62].

In this study, we immobilized covalently a biosignaling molecule (a growth factor) on dopamine-treated metal surfaces. We aimed to optimize the conditions of dopamine treatment that are suitable for surface modification by providing an immobilization site, such as an amino group, and investigated quantitatively the effect of immobilization of a growth factor, the epidermal growth factor (EGF), on metal surfaces.

## 2. Materials and methods

### 2.1. Materials

The rat kidney adherent fibroblast cell line NRK49F was provided by the RIKEN Cell Bank (Tsukuba, Japan) and was maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 5% fetal bovine serum (FBS; Moregate Inc., Hamilton, Waikato, New Zealand) and 1% penicillin-streptomycin, purchased from Wako Pure Chemical Industries (Osaka, Japan). Trypsin containing 1 mM ethylenediaminetetraacetic acid (EDTA) was purchased from Wako Pure Chemical Industries. Recombinant human EGF was obtained from R&D Systems Inc. (Minneapolis, USA).

3,4-Dihydroxyphenethylamine hydrochloride (dopamine) and *N*-hydroxysuccinimide (NHS) were purchased from Wako Pure Chemical Industries. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (water-soluble carbodiimide, WSC) was obtained from Dojindo (Kumamoto, Japan).

The monoclonal anti-human EGF antibody was purchased from R&D Systems Inc. The fluorescein isothiocyanate (FITC)-conjugated secondary antibody was obtained from Cappel Research Reagents (Costa Mesa, USA). Block Ace Powder was obtained from DS Pharma Biomedical (Sapporo, Japan).

A glass plate (diameter, 15 mm; thickness, 1 mm) was coated with titanium by Osaka Vacuum Ind. Co. (Osaka, Japan). The glass plate was cleaned via nine rounds of ultrasonication in ultrapure water and was dried using heated gas. Pure titanium was vacuum deposited on the plate using an electron beam with a thickness of 400 nm ( $\pm 25\%$ ). Tissue-culture polystyrene plates with 24 wells (well diameter, 16 mm) were purchased from BD Falcon (New Jersey, USA) and 48-well plates were obtained from Corning (New York, USA). Stainless steel (SUS316; diameter, 10 mm; thickness, 1 mm) was purchased from Iwasaki Co. (Osaka, Japan).

### 2.2. Surface treatment

The surface of the plates was washed in hexane solution, cleaned with 6 M hydrogen chloride for 10 min, rinsed twice with triple distilled water, dried in a vacuum oven for 24 h, and cleaned photochemically

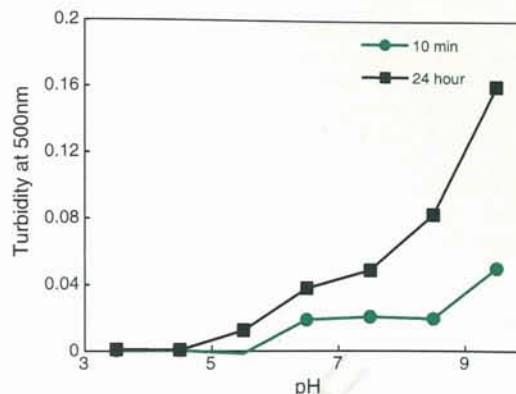


Fig. 1. Turbidity of dopamine solutions at different pH values.

using an excimer UV lamp (USHIO Inc., Tokyo, Japan) for 10 min before incubation in the solution of dopamine. This method was applied to achieve complete removal of C–C bonds and avoid subsequent decomposition of the organic molecules [5,7]. The complete removal of the organic material was confirmed by the decrease of the water contact angle, a value of  $\theta = 0^\circ$  being indicative of a surface that is absolutely hydrophilic.

Dopamine treatment was performed thereafter. The cleaned plates were placed in a flask containing 1 or 2 mg/mL of dopamine solution in water (the resulting pH was 4.5) or 10 mM Tris-buffer (adjusted to pH 8.5). The reaction was performed at room temperature for 24 h. The dopamine-treated  $\text{TiO}_2$  and SUS316 plates were rinsed in fresh water, followed by drying in a clean vacuum oven at room temperature for 24 h.

For immobilization of EGF on the surface of the plates, an EGF solution was mixed with an aqueous solution of 50 mM of WSC and 20 mM of NHS. The dopamine-treated plates were immersed in the mixed solution for 48 h at  $4^\circ\text{C}$  [40]. After the incubation, the plates were washed three times with phosphate-buffered saline (PBS).

### 2.3. Surface analysis

The static water contact angles of the sample surfaces were measured at  $25^\circ\text{C}$  in air using a contact-angle meter (Kyowa Interface Science Co., Tokyo, Japan) based on the sessile drop method. All contact angles were determined by averaging 10 different point values measured on each dopamine-treated surface.

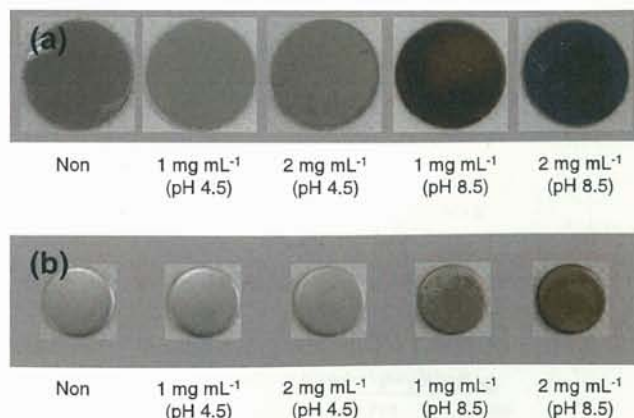


Fig. 2. Images of dopamine-treated titanium (a) and stainless steel (b) materials.

**Table 1**  
Water contact angle and thickness of the dopamine layer formed on the metal surface.

pH	Dopamine concentration	Water contact angle		Thickness
		On titanium	On stainless steel	On titanium
4.5	1 mg/ml	46.82 ± 1.07°	52.67 ± 3.51°	3.40 ± 0.49 nm
	2 mg/ml	50.71 ± 0.64°	58.76 ± 1.66°	3.42 ± 0.64 nm
8.5	1 mg/ml	46.92 ± 0.84°	41.23 ± 3.40°	3.36 ± 0.55 μm
	2 mg/ml	48.82 ± 0.79°	46.78 ± 1.79°	4.97 ± 0.63 μm

The surfaces were also analyzed using X-ray photoelectron spectroscopy (XPS; AXIS-HS, Kratos, Manchester, UK) in an *in vacuo* condition of less than  $10^{-7}$  Pa. A *K*- $\alpha$  monochromatic X-ray with a source power of

150 W was used. Wide and narrow scans were acquired at a pass energy of 80 and 40 eV, respectively. Overview spectra were obtained in the range of 0–1100 eV using an analyzer pass energy of 80 and 40 eV.

The thickness of the polymer was measured using an ellipsometer M-2000DI (JA Woollam Company, Nebraska, USA) in the spectral range of 195–1500 nm using three angles (65°, 70°, and 75°). The roughness of the surface was analyzed using a New View 5032 apparatus (Zygo Co., Middlefield, USA). The surface was observed by a scanning electron microscope (SEM, JSM6330F, JOEL Ltd., Akishima, Japan) without any treatments.

FITC was used to determine the amount of amino groups on surfaces. One hundred microliters of a 10 mg/mL FITC solution in dimethylsulfoxide was mixed with 1 mL of 0.1 M sodium bicarbonate

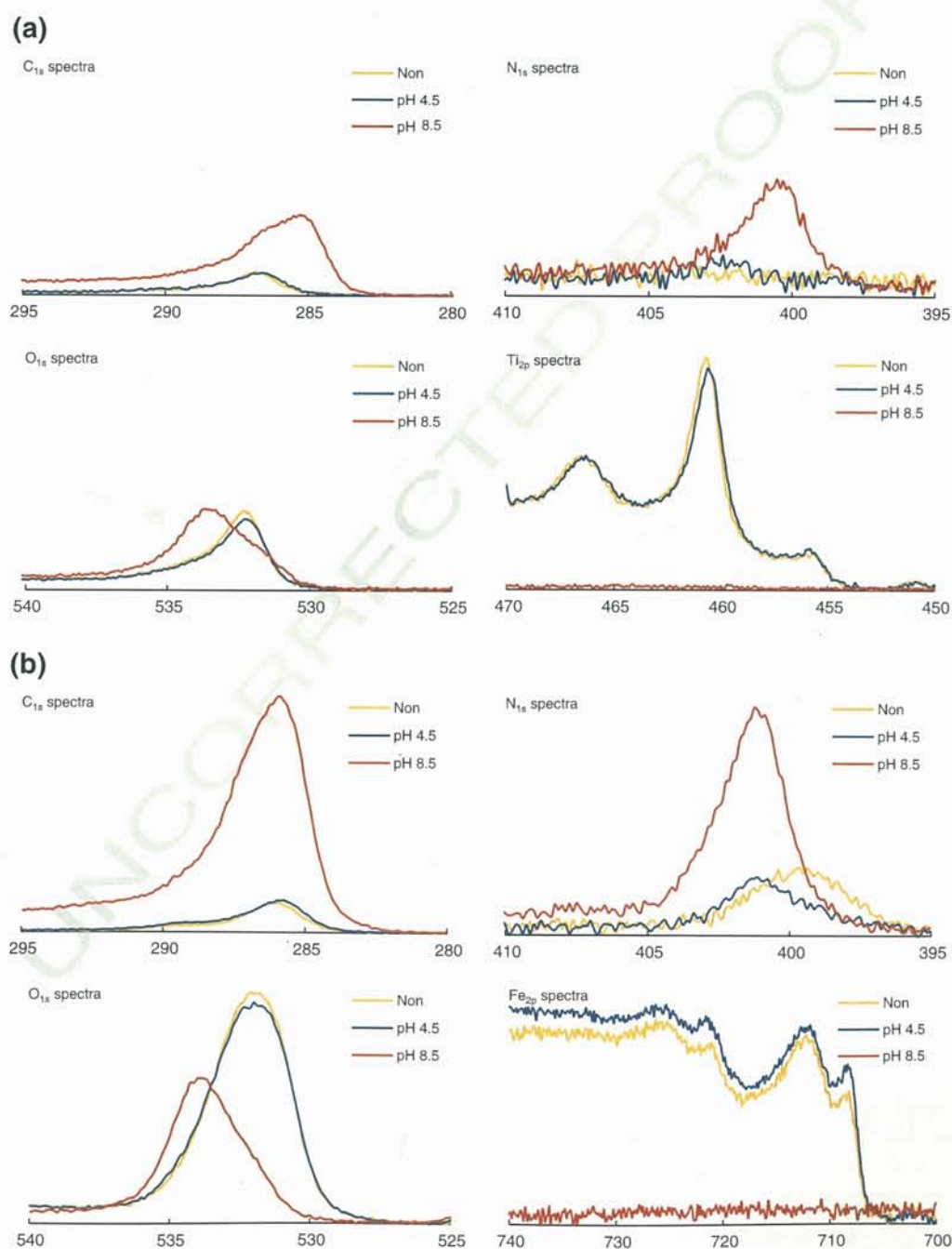


Fig. 3. XPS spectra of titanium (a) and stainless steel (b).



solution (pH 9.0). The sample plate was incubated in the solution for 1 h at room temperature and rinsed with PBS 10 times. The amount of FITC was measured using an AxioVision instrument (Zeiss, Oberkochen, Germany) with a Cool SNAP HQ camera (Photometrics, Tokyo, Japan).

The amount of immobilized EGF was determined using an anti-EGF antibody. The plate was rinsed with PBS–Tween (0.2%) and blocked by incubation in an aqueous solution of 1% nonfat milk for 30 min. Subsequently, the plate was incubated with an anti-EGF antibody (diluted 1000 times) overnight at 4 °C and washed three times with PBS–Tween (0.2%) before incubation with an FITC-conjugated secondary antibody (diluted 1000 times) for 1 h at room temperature. After washing three times with PBS–Tween (0.2%), the amount of FITC was measured using an AxioVision instrument with a Cool SNAP HQ camera.

To make calibration curve, various concentrations of FITC-conjugated secondary antibody were spotted on the surface.

#### 2.4. Cell culture

NRK49F cells were cultured in DMEM supplemented with 5% FBS and 1% penicillin–streptomycin at 37 °C in 95% humidified air/5% CO<sub>2</sub>. The cells were then washed using 5 mL of PBS and harvested using a 0.25% trypsin solution containing 1 mM EDTA for 3 min at 37 °C. Finally, the recovered cells were suspended in medium for the following *in vitro* examination. The cell suspension was added to 24- or 48-well tissue culture polystyrene plates (1.0 mL/well,  $5 \times 10^3$  cells/mL) containing the samples, which had been washed previously with sterilized PBS. The cells were

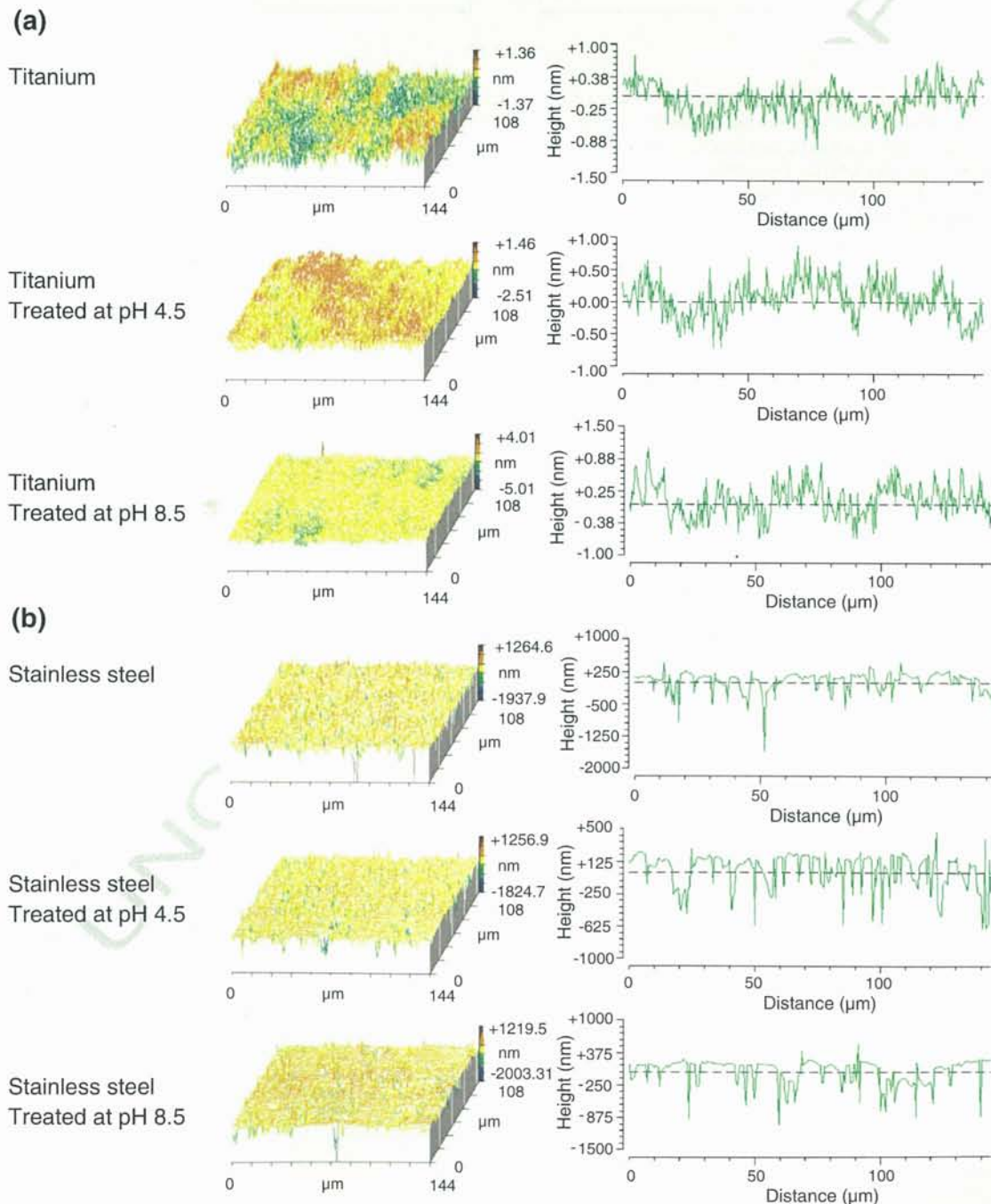


Fig. 4. Surface roughness of dopamine-treated and nontreated titanium (a) and stainless steel (b). (c) Illustration of the roughness parameters,  $R_a$  and  $R_q$ .



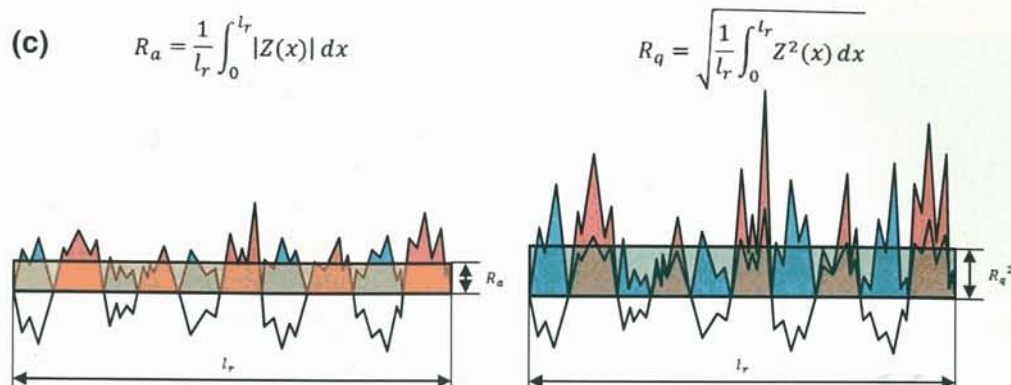


Fig. 4 (continued).

cultured under a 5% CO<sub>2</sub> atmosphere at 37 °C for 5 days and were studied using a phase-contrast microscope. The number of cells was counted using the microscope. Statistical analyses were performed using analysis of variance.

### 3. Results

#### 3.1. Appearance and measurement of the water contact angle

First, the properties of the dopamine solution were investigated based on turbidity, as shown in Fig. 1. The solution was transparent at low pH, even after 24 h, and turned to turbid and brown at high pH. After 24 h, some precipitate was found at high pH. In this study, dopamine treatment was performed at pH 4.5 or 8.5, which were used as typical low and high pH values, as described by He et al. [40] and Lee et al. [45], respectively. Because the concentration of the solutions used by He et al. [40] and Lee et al. [45] were 1 and 2 mg/mL, respectively, we also used these two concentrations.

The surface of the metals turned brown when the titanium and stainless steel were treated at pH 8.5 (Fig. 2). In contrast, no significant color change was observed when they were treated at pH 4.5 (Fig. 2). However, the assessment of surface hydrophilicity based on contact angle measurements revealed that the water contact angle of surfaces increased with dopamine treatment, regardless of the type of metal or pH (Table 1). This indicates that the surfaces of titanium and stainless steel were fully covered by dopamine, regardless of pH. Li et al. [57] and Wei et al. [58] reported contact angles of a polydopamine-treated surface of 51 ± 1.5° and ~55°, respectively. The static water contact angles of the treated surfaces analyzed in the present study were similar to these values.

#### 3.2. XPS analysis, ellipsometry analysis, roughness measurement, SEM image, and measurement of amino groups

XPS analysis was performed on titanium and stainless steel surfaces treated with dopamine at different pH values (Fig. 3). C<sub>1s</sub> and N<sub>1s</sub> spectra

demonstrated that significant peaks corresponding to an organic layer appeared on titanium and stainless steel surfaces after treatment at pH 8.5. O<sub>1s</sub> spectra indicated that a chemical shift of oxygen was observed on titanium and stainless steel surfaces only after treatment at pH 8.5. Ti<sub>2p</sub> and Fe<sub>2p</sub> spectra showed that the metal surfaces were not detectable by XPS after treatment at pH 8.5. These results indicate that a thick layer of dopamine was formed on the metal surfaces treated with dopamine at pH 8.5, as the probing depth of the XPS technique is about 8 nm in an organic matrix [44,62].

This result was confirmed by thickness measurement using ellipsometry, as shown in Table 1. Surface roughness was also measured, as shown in Fig. 4 and summarized in Table 2. The surface of the stainless steel material used in this study exhibited a degree of roughness that precluded the measurement of the thickness of the layer formed (Table 2). In contrast, the thickness of the layer covering the titanium surface could be measured: the thickness of the dopamine layer formed at pH 8.5 was about 1000 times as thick as that formed at pH 4.5. The roughness of the metal increased slightly after treatment at pH 8.5. For the stainless steel material, this variation was not significant because of its high degree of surface roughness, although the surface roughness of the stainless steel material decreased slightly after dopamine treatment.

The surface was also observed by SEM as shown in Fig. 5. The surface of stainless steel was rougher than that of titanium. The dopamine treatment reduced the roughness and the treatment at pH 8.5 reduced more than that at pH 4.5. Microphotos with high magnification show nano-particle deposition on the surfaces at pH 8.5 as reported by Jiang et al. [63].

The amount of amino groups present in the dopamine layer was measured using FITC (Fig. 6). The amount of amino groups in the organic layer formed at pH 8.5 was higher than in that formed at pH 4.5, for both titanium and stainless steel materials. However, the variation in amino group content was smaller than that observed for thickness (Table 1). It is known that amino groups in dopamine are consumed by polymerization at pH 8.5. Therefore, this result suggests that some amino groups remained available for reactivity, even after the polymerization of dopamine. The differences observed for the two metals were considered as being caused by differences in roughness. The surface of the stainless steel material was so rough that the apparent surface area was larger and the amount of amino acids produced was higher than that observed on the smoother titanium surface.

#### 3.3. Immobilization of EGF

On both metals, the amount of immobilized EGF increased with the increase of EGF in solution, as shown in Fig. 7. In addition, the amount of EGF immobilized on surfaces treated with dopamine at pH 8.5 was higher than on those treated at pH 4.5, for both metals. There were no significant differences in the degree of EGF immobilization between

Table 2

Roughness of the surface of the titanium and stainless steel materials.

	Surface treatment with dopamine	$R_a$ (nm) <sup>a</sup>	$R_q$ (nm) <sup>a</sup>
Titanium	No	0.27 ± 0.01	0.34 ± 0.01
	pH 4.5	0.27 ± 0.01	0.34 ± 0.01
	pH 8.5	0.34 ± 0.03	0.45 ± 0.06
Stainless Steel	No	175 ± 4.99	236 ± 6.93
	pH 4.5	155 ± 3.35	216 ± 3.14
	pH 8.5	157 ± 0.48	220 ± 0.02

<sup>a</sup>  $R_a = \frac{1}{l_r} \int_0^{l_r} |Z(x)| dx$  and  $R_q = \sqrt{\frac{1}{l_r} \int_0^{l_r} Z^2(x) dx}$

(a)

Titanium

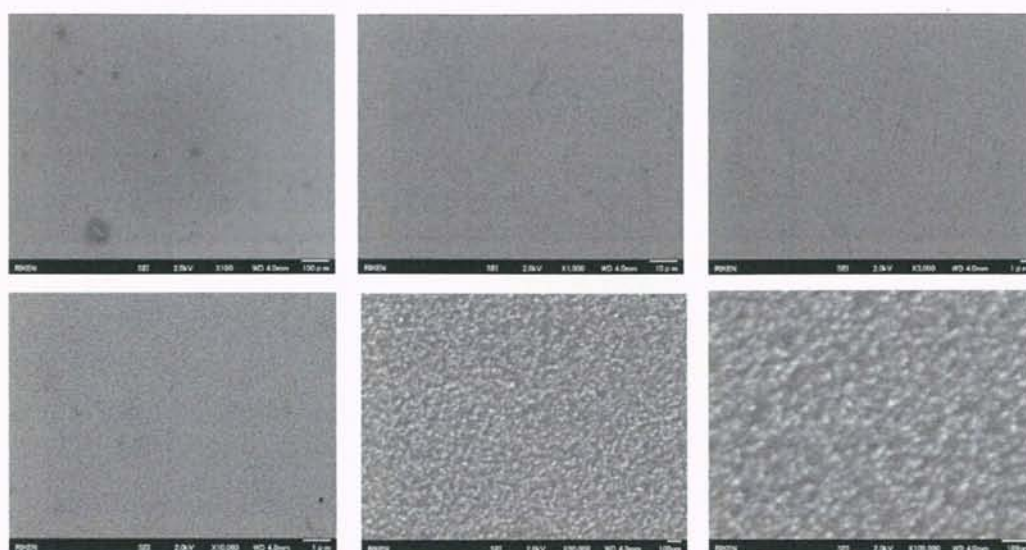
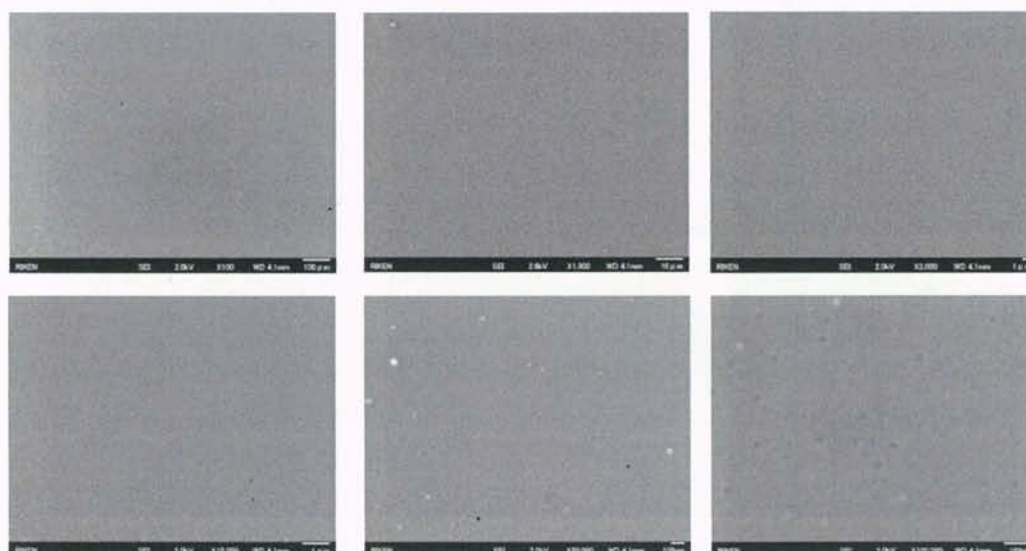
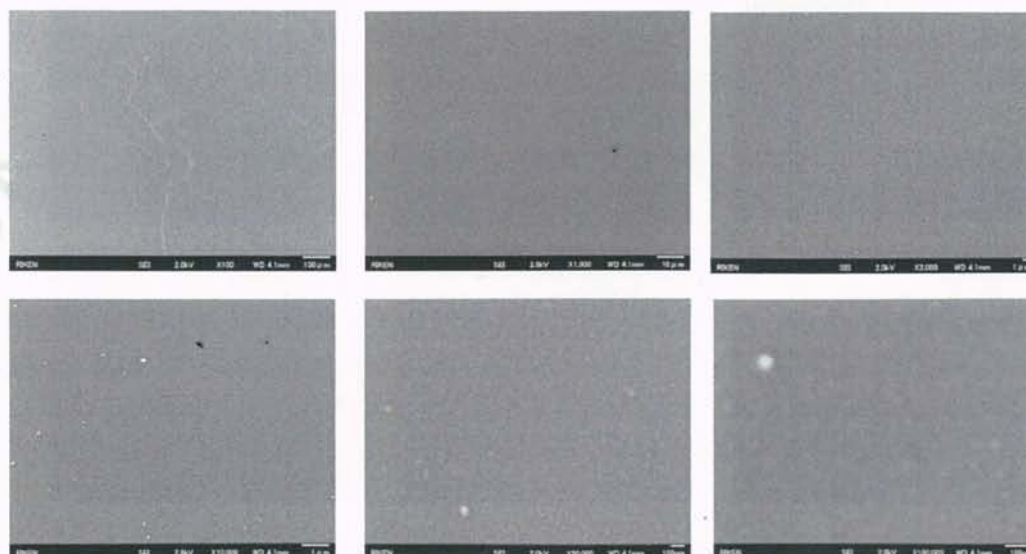
Titanium  
Treated at pH 4.5Titanium  
Treated at pH 8.5

Fig. 5. SEM images of dopamine-treated and nontreated titanium (a) and stainless steel (b).



(b)

Stainless steel

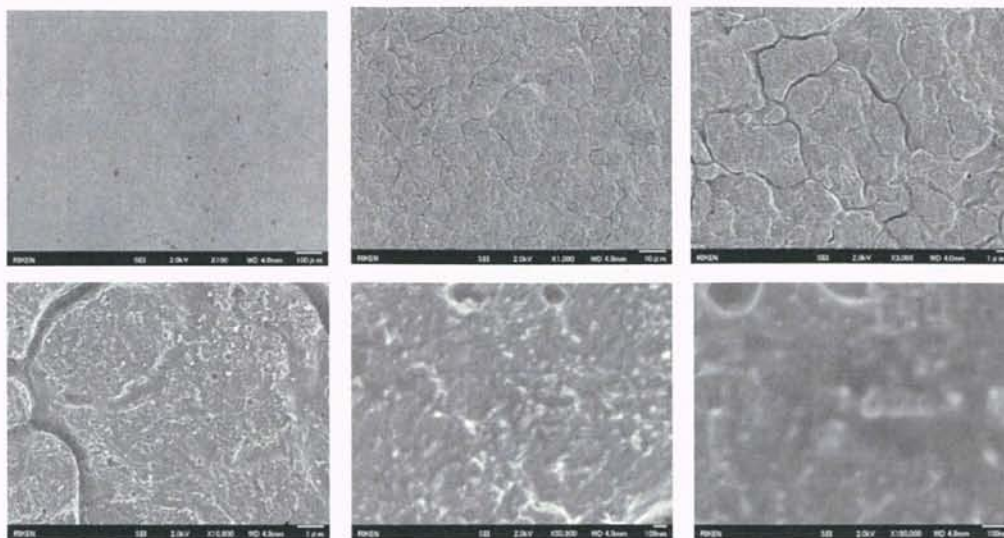
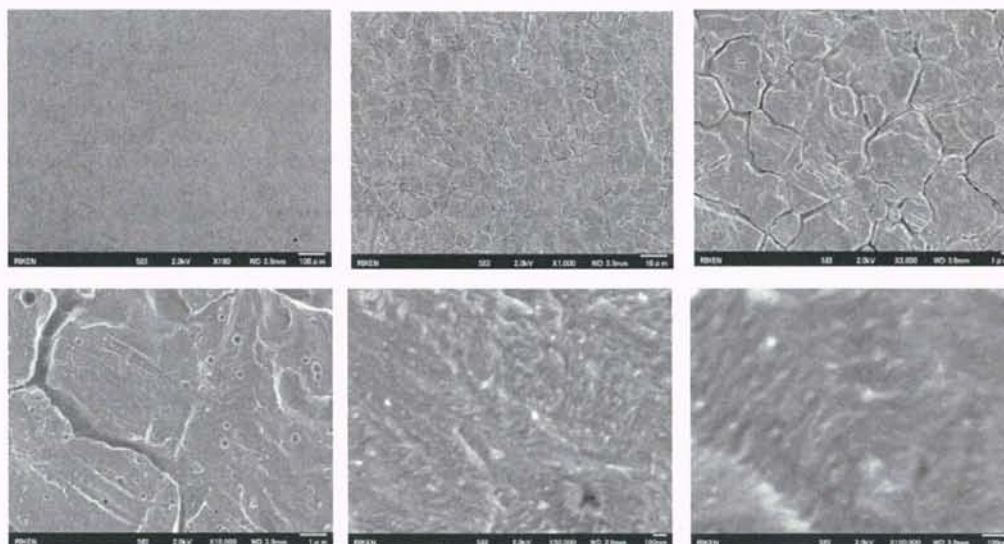
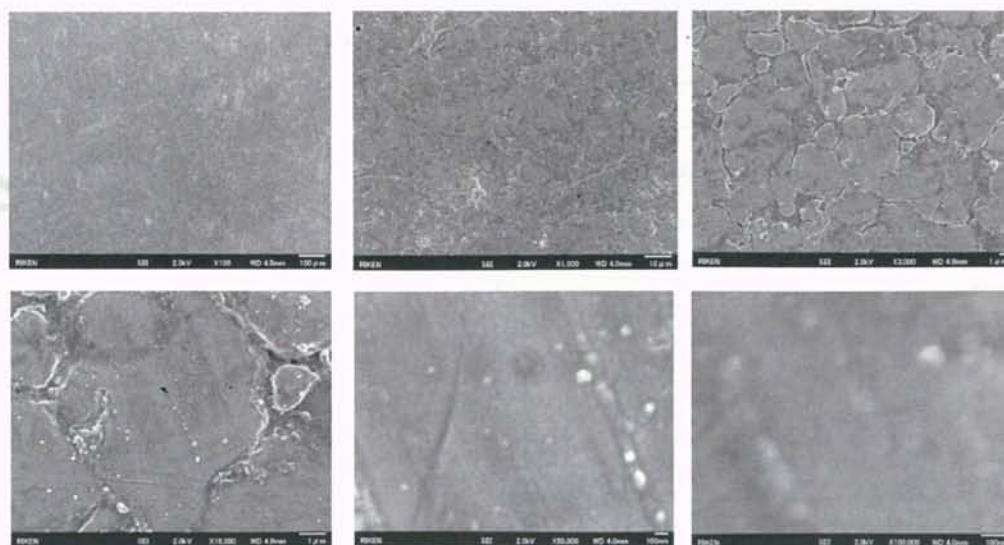
Stainless steel  
Treated at pH 4.5Stainless steel  
Treated at pH 8.5

Fig. 5 (continued).

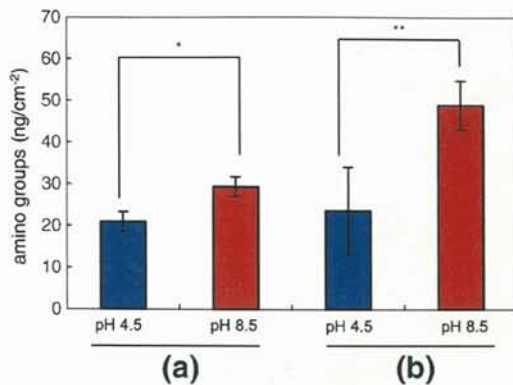


Fig. 6. Amount of amino groups on dopamine-treated titanium (a) and stainless steel (b) surfaces ( $n=5$ ;  $p^*<0.005$ ;  $p^{**}<0.05$ ).

titanium and stainless steel. This result indicates that, after dopamine treatment, the surfaces of these metals were similar to each other.

### 3.4. Cell proliferation on EGF-immobilized surfaces

Fig. 8 shows the growth of NRK49F cells in the presence of soluble and immobilized EGF. Cell growth was enhanced with the increase of the amount of EGF in a well. Immobilized EGF enhanced cell growth efficiently compared with soluble EGF, on both metals. Lower amounts of immobilized EGF were sufficient to enhance cell growth compared with soluble EGF. In addition, the maximum enhancement effect of immobilized EGF was higher than that of soluble EGF. These phenomena were observed on several polymer surfaces, as reviewed previously [4,18,19]. The present investigation confirmed the effectiveness of the immobilization of a growth factor on surface-treated metals.

In addition, there were no significant differences between titanium and stainless steel regarding the effect of immobilized vs soluble EGF. This result also indicates that, after dopamine treatment, the surfaces of the two metals were similar to each other.

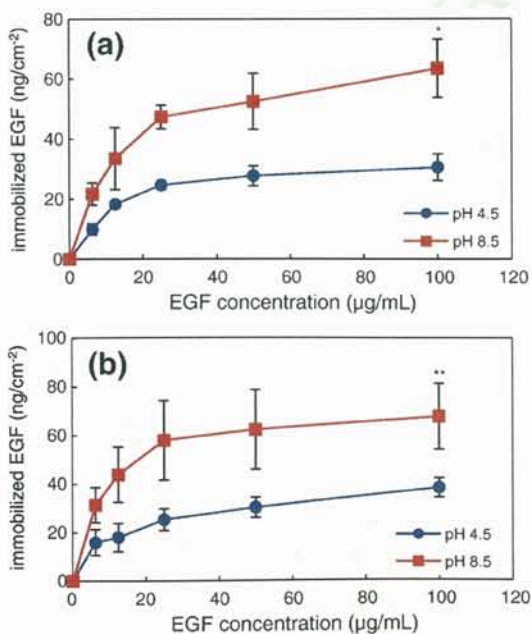


Fig. 7. Amount of EGF immobilized on titanium surfaces treated with dopamine at pH 4.5 (—●—) and at pH 8.5 (—■—) (a) and stainless steel surfaces treated with dopamine at pH 4.5 (—●—) and at pH 8.5 (—■—) (b) ( $n=3$ ;  $p^*<0.05$ ;  $p^{**}<0.05$ ).

## 4. Discussion

### 4.1. Surface treatment with dopamine

Mussels are promiscuous fouling organisms that attach to virtually all types of inorganic and organic surfaces. The identification of dopamine as a small-molecule compound that contains both functionalities, by Lee et al. [45], led to the assumption that the coexistence of catechol (DOPA) and amine (lysine) groups is crucial to achieve adhesion to a wide spectrum of materials. The formation of polydopamine or dopamine-melanin has been described and is used for surface modification under mild alkali conditions. Simple immersion of substrates in a diluted aqueous solution of dopamine buffered to a pH typical of marine environments (2 mg of dopamine per mL of 10 mM Tris, pH 8.5) resulted in spontaneous deposition of a thin layer of adherent polymer. Protein immobilization was performed by some researchers on dopamine-treated surfaces. Lee et al. immobilized trypsin on dopamine-treated surfaces via a reaction between nucleophiles and the polydopamine surface [56]. Similarly, Poh et al. immobilized the vascular endothelial growth factor on dopamine-treated titanium [17]. Yang et al. immobilized avidin on a polydopamine surface [51], and Wei et al. immobilized bovine serum albumin on dopamine-treated polymeric membranes [58].

Conversely, at low pH, the formation of polydopamine does not occur as shown in Fig. 1. However, Xu et al. [39] and He et al. [40] treated iron oxide or titanium surfaces with dopamine dissolved in pure water (the resulting pH was about 4.5), respectively, using the binding affinity of the catechol structure. He et al. immobilized RGD peptide or collagen on dopamine-treated titanium [40]. To date, no comparison of the effect of pH on the dopamine treatment has been performed. Therefore, in this study, we investigated the effect of pH on the treatment of metal surfaces using dopamine. The results of this analysis are illustrated in Fig. 9. At low pH, a dopamine monolayer was formed on the titanium and stainless steel materials; however, the amount of immobilized EGF was higher at high pH. As a thicker layer was formed at high pH, the apparent increases of surface roughness and content of amino groups were considered.

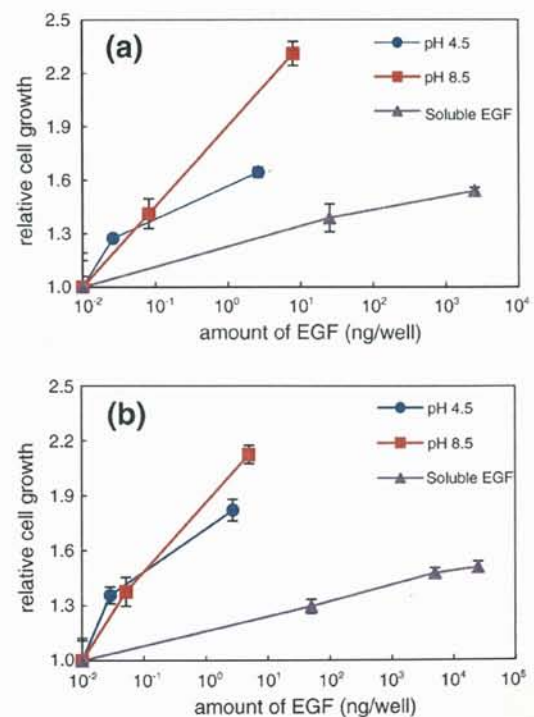


Fig. 8. Growth of NRK49F cells on an EGF-immobilized titanium surface treated with dopamine at pH 4.5 (—●—) and at pH 8.5 (—■—) (a) and on a stainless steel surface treated with dopamine at pH 4.5 (—●—) and at pH 8.5 (—■—) (b) ( $n=3$ ).



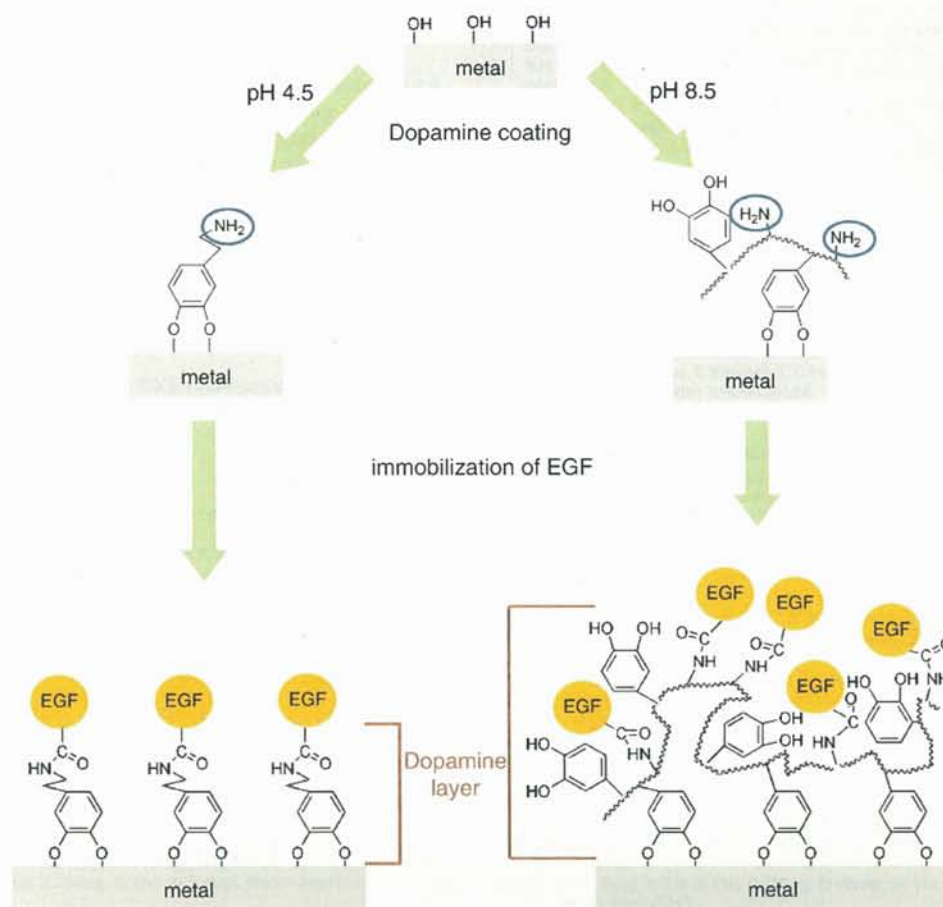


Fig. 9. Schematic illustration of the treatment of a metal surface using dopamine.

The interaction between the dopamine layer and the metal include covalent and non-covalent interactions. The former includes the reactions of *o*-quinone yielded by oxidation of catechol with catechol by quinone-phenol dismutation, or with amine by Michael addition and Schiff base reaction [64]. The latter includes the hydrogen bonding interaction,  $\pi$ – $\pi$  interaction, and electrostatic interactions. However, the polymerization mechanism and deposition behavior of dopamine on various substrates are in dispute and not yet clearly known. The possible structure evolution of dopamine in aqueous solution was considered by Jiang et al. [63] recently. According to their consideration, dopamine is easily oxidized by dissolved oxygen under alkaline conditions, creating 5,6-dihydroxyindole and 5,6-indolequinone via intramolecular cyclization, oxidation, and rearrangement. After the multistep reaction proceeding by these compounds, a mass of melanin-like dopamine aggregates was generated in the solution and a tightly adherent dopamine layer was formed on the surfaces of the substrates simultaneously.

#### 4.2. Immobilization of EGF

Regarding the amount of immobilized EGF, different surface densities have been reported. Ito et al. [65], Chen et al. [66], Nakaji-Hirabayashi et al. [67], Liberelle et al. [68], Klenkler et al. [69], and Gonçalves et al. [70] reported EGF surface densities of 90 ng/cm<sup>2</sup>, 0.2 EGF molecules/nm<sup>2</sup> (200 ng/cm<sup>2</sup>), 168–380 ng/cm<sup>2</sup>, 14–30 pmol/cm<sup>2</sup> (84–180 ng/cm<sup>2</sup>), 300 ng/cm<sup>2</sup>, and 4–18 ng/cm<sup>2</sup>, respectively. Because of the difficulties in the evaluation of surface area, which depends on the roughness of the surface, it is not possible to compare the surface concentrations reported. However, considering that the densely packed monolayer of immobilized EGF ( $2.97 \times 10^{-13}$  cm<sup>2</sup>) [71] theoretically

provides about 31 ng/cm<sup>2</sup> of surface concentration, the maximum surface concentration of immobilized EGF in the layer formed at pH 4.5 indicates the formation of an EGF monolayer. The organic layer formed on another surface prepared at pH 8.5 was thick and the surface was considered as rough. The roughness increased the surface area on which EGF could be immobilized.

#### 4.3. Effect of immobilized EGF

After the demonstration that immobilized insulin enhanced cell growth significantly, EGF was also immobilized on surfaces. The effect of immobilized growth factors was discussed by Ito [4,18,19]. The effectiveness of immobilized growth factors was confirmed by micropattern immobilization, antibody blocking, radioisotope labeling, microarray-based comparison with other proteins, etc. [4,18,19]. It is known that the effect of immobilized growth factors is higher than that of soluble ones. [18,19,72]. In addition, immobilized EGF induced some effects different from those of soluble EGF on some cells [18,19,73]. The results of the present study demonstrated that a lower amount of immobilized EGF was sufficient for cell growth and that its maximum enhancement effect was higher than that of soluble EGF, on both titanium and stainless steel surfaces. This observation can be explained mainly by the following factors: the high local concentration of growth factors and the multivalency of immobilized growth factors. In addition, the main reason for the observation of a higher activity of immobilized EGF vs soluble EGF may be the inhibition of downregulation, considering recent reports on long-lasting activation by immobilized EGF [4,18,19,65,73].

## 5. Conclusions

Metal (titanium and stainless steel) surfaces were treated via a biomimetic method using dopamine, and a growth factor (EGF) was immobilized on the treated surfaces. At high pH, a dopamine–melanin layer was formed on the surface of these metals that provided a larger amount of amino groups for coupling with EGF. Immobilized EGF promoted cell growth more efficiently than soluble EGF. Surface modification methods will provide new biologically functional metals that can be used for the development of medical devices.

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