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Article Type: Invited Review Article

Keywords: Titanium implants, Surface modification, Bisphosphonate, Simvastatin, Peptides

Abstract: Since biomaterials contact many different tissues, those materials must have optimum surface compatibility with the host bone tissue and soft tissue, as well as anti-microbial properties on an exposed region of the mucosa. Such materials can be created under well-controlled conditions by modifying the surfaces of materials that contact those tissues. This paper is focused on the surface modification of biomaterials for developing “Bio-functional dental implants”, which are compatible with all host tissues, using a cold-plasma technique. At the bone tissue/implant interface, a thin calcium phosphate coating and rapid heating with infrared radiation were effective in controlling the dissolution without cracking the coating. These thin calcium phosphate coatings may directly promote osteogenesis, but also enable immobilization and subsequent drug delivery system (DDS) of bisphosphonates. Simvastatin is also an effective candidate that is reported to increase the expression of BMP-2. The thin-film of hexamethyldisiloxane (HMDSO) was plasma-polymerized onto titanium, and then HMDSO surface was activated by O2-plasma treatment. A quartz crystal microbalance (QCM-D) technique demonstrated that simvastatin was immobilized on the plasma-treated surfaces due to introduction of O2-functional groups. At the soft tissue/implant interface, multi-grooved surface topographies and utilizing the adhesive proteins such as fibronectin or laminin-5 may help in providing a biological seal around the implant. At the oral fluid/implant interface, an alumina coating, F+-implantation and immobilization of anti-microbial peptides were responsible for inhibiting the biofilm accumulation.
Surface modification by cold-plasma technique for dental implants

- Bio-functionalization with binding pharmaceuticals-

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Running title: Surface modification using cold-plasma technique
Thank you for your valuable suggestions,
I have revised the manuscripts as follows according to the reviewers.

1. "Conflict of interest statement" was added.
2. Tables were re-arranged correctly (I am sorry to miss the Tables).
3. I explained more about the "cold-plasma technique" itself.
4. Number of figures was reduced to 22.
5. “DDS” and "QCM-D” were spelled out.
6. Mistakes in text and figure legends were revised.

Sincerely yours,
Masao Yoshinari
Surface modification by cold-plasma technique for dental implants

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Abstract

Since biomaterials contact many different tissues, those materials must have optimum surface compatibility with the host bone tissue and soft tissue, as well as anti-microbial properties on an exposed region of the mucosa. Such materials can be created under well-controlled conditions by modifying the surfaces of materials that contact those tissues. This paper is focused on the surface modification of biomaterials for developing “Bio-functional dental implants”, which are compatible with all host tissues, using a cold-plasma technique.

At the bone tissue/implant interface, a thin calcium phosphate coating and rapid heating with infrared radiation were effective in controlling the dissolution without cracking the coating. These thin calcium phosphate coatings may directly promote osteogenisis, but also enable immobilization and subsequent drug delivery system (DDS) of bisphosphonates. Simvastatin is also an effective candidate that is reported to increase the expression of BMP-2. The thin-film of hexamethyldisiloxane (HMDSO) was plasma-polymerized onto titanium, and then HMDSO surface was activated by O_2-plasma treatment. A quartz crystal microbalance (QCM-D) technique demonstrated that simvastatin was immobilized on the plasma-treated surfaces due to introduction of O_2-functional groups. At the soft tissue/implant interface, multi-grooved surface topographies and utilizing the adhesive proteins such as fibronectin or laminin-5 may help in providing a biological seal around the implant. At the oral fluid/implant interface, an alumina coating, F^+-implantation and immobilization of anti-microbial peptides were responsible for inhibiting the biofilm accumulation.
Key words:

Titanium implants, Surface modification, Bisphosphonate, Simvastatin, Peptides
1. Introduction

The vital reaction of biomaterials is affected by the “surface topography” and “surface physico-chemistry” of the materials. Surface topography has marked effects on cell behavior. Generally, cell adhesion is greater on rough surfaces than on smooth surfaces, but the actual rate of adhesion depends on the type of cell. Contact guidance, the phenomenon in which cells align along grooves of the substrate, is one example of surface topography controlling cell behavior (1,2). Surface roughness alters osteoblast proliferation, differentiation, and matrix production in vitro, and plays a role in determining the phenotypic expression of cells in vivo (3, 4). Surface topography is controlled by machining, blasting, acid etching, or laser lithography.

Surface physico-chemistry involves the adsorption of proteins, bacteria, and cells on biomaterials. This adsorption reflects the affinity between two substances. Adsorption characteristics are primarily influenced by hydrophobicity (wettability), which can be determined by measuring the surface energy (hydropathy index), and electrokinetic potential (zeta potential, isoelectric point), which reflects surface electric charges according to the isoelectric point (x-axis in Fig.1) and hydropathy index (y-axis in Fig.1) of the amino acids in the protein (Fig.1).

A cold plasma-surface modification (Fig.2) is suitable for change in surface physico-chemistry, which involves dry process including ion implantation, ion plating, ion sputtering, ion beam dynamic mixing, plasma polymerization and plasma treatment with partly ionized gases, which is generated in a high-voltage electric field in a low pressure. This approach is superior in that it is environmentally pollution-free, it yields safe products, and good quality control can be maintained, thus ensuring defect-free films. In this process, substrates and targets are placed in a vacuum chamber, a vacuum is created and the coating materials are then deposited onto the substrates in a cold plasma atmosphere.
Fig. 3 shows the relationship between film thickness and energy of atoms, which is related to adherence of coatings, in various coatings produced by ion beam techniques. It can be seen that the thinner the coatings, the higher the degree of adherence. Surface modification using a cold plasma has advantages; 1) Thin and porosity-free coatings, 2) Control of surface energy and surface electric charge, 3) Introduction of functional groups, 4) Cleaning of surfaces, 5) Graft-polymerization and adhesion, 6) Etching and micro-patterning and 7) Application for drug delivery system.

This review will describe the surface modification of titanium implant for bio-functionalization (Fig.4) (5) related to the surface topography and physico-chemistry of different host tissues.

2. Bone tissue / Implant interface

The integration of bone tissue with an implant requires a rough surface topography that maximizes the area in contact with the bone and allows the contact guidance for the development of osteoblasts. As for surface physico-chemistry, surface modification with a thin calcium phosphate coating is useful in producing rapid osteogenesis and strong osseointegration. There have been also attempts to employ drugs such as bisphosphonate and simvastatin, which induce osteogenesis more efficiently.

2.1 Thin calcium phosphate (CaP) coating

CaP implants, including hydroxyapatite (HAp), are well known for good osteoconductivity (the early stage of osteogenesis) as well as for direct binding to bone tissue in vivo. Alkaline phosphatase expression and parathyroid hormone response were higher in cultures grown in HAp than in cultures grown in titanium (6), and the in vitro formation of extracellular matrices was greater on CaP coatings
than on titanium. Several mechanisms of the principal factors involved in osteogenesis on CaP ceramics have been considered. First, implanted CaP (HAp) acts as a nucleation site and exhibits crystallographic properties in an epitaxial process of the newly developed structure. The calcium ions dissolve from the CaP surface, resulting in the deposition of a mineralized layer. This stimulates the bone cells to continue extracellular matrix (bonding zone) synthesis and calcification (7). CaP ceramics adsorb many osteo-conductive and/or osteo-inductive proteins, which have an important role in the mineralization of bone tissues.

In spite of their rapid and strong bonds to living bone tissues and favorable osteogenic ability, CaP ceramics alone cannot be used for implants because of their lack of strength. Accordingly, CaP coatings on Ti implants produced by the plasma spraying have frequently been used (8). These CaP coated implants, however, often develop fractures in their coatings as well as at the titanium interface after implantation. The reason for this is thought to originate in the comparatively thick, porous, non-uniform (crystalline surrounded by an amorphous mass), and poorly adherent CaP layer produced by plasma spraying (Fig. 5). These fragments of a certain size cause phagocytosis by macrophages, leading to inflammation. It is therefore desirable for the materials to be rapidly and completely absorbed in the host tissues and to be entirely replaced with bone tissue. When osteogenesis occurs at the site where old bones are absorbed (remodeling of bones), the CaP coatings should be no thicker than necessary.

Attempts have recently been made to solve problems in the plasma spraying technique using a cold-plasma surface modification as above-mentioned (9). In the cold plasma, ion-plating (10) and the ion sputtering (11), which are a kind of physical vapor deposition (PVD), are used to produce implant materials consisting of a thin, homogeneous, and adherent CaP coating. Ion beam dynamic mixing (IBDM) was also introduced as a suitable technique for fabricating a thin and adherent ceramic layer (12). This method is a combination of ion implantation and PVD,
and has the advantages of a high deposition rate, producing defect-free transparent thin films, and excellent adhesion compared to conventional thin-film deposition techniques.

Fig. 6 shows a commercially available Brånemark implant and a CaP coated implant. The slight color change is recognized in the coated implant due to the thin (1 µm) and defect-free coating. Good degrees of osteogenesis and bond strength with bone are obtained in the thin CaP coated implants (13).

Although as-deposited coatings were confirmed to be amorphous by thin X-ray diffraction (XRD) analysis, post-deposition heat treatment was reported to result in a change in crystallinity (9,11,14,15,16,17,18,19). The diffraction pattern of heat-treated coatings showed mainly HAp, with a preferential orientation of the c-axis (002) (Fig.7) (17). Fourier transformation infrared (FT-IR) spectrometry analysis has revealed absorption of OH\(^{-}\) and PO\(_4\)\(^{3-}\) in both as-deposited and heat-treated coatings (18). The binding energies of P2p, Ca2p3/2 and O1s obtained from coated specimens were close to those of HAp bulk, according to X-ray photoelectron spectroscopy (XPS) (11).

2.2. Change in bond strength and solubility by heat treatment

The tensile bond strength of as-deposited coatings to Ti substrates has been reported to be 38-45 MPa with ion beam sputter deposition (20), more than 53 MPa with magnetron sputtering (10) and in excess of 59 MPa with IBDM (11). Thus, thin CaP coatings produced by a cold plasma system demonstrate high bond strength to Ti substrates, as they have fewer defects than, and offer superior adhesion to, those produced by conventional plasma spraying methods.

Unfortunately, as-deposited coatings are amorphous (Fig.7a), resulting in films that easily dissolve in body fluids (17). This renders them inappropriate for biomedical use. As-deposited coatings crystallize during heat treatment using a conventional electric furnace (Fig.7b), leading
to decreased solubility (13, 14, 17, 19). Such coatings, however, tend to crack easily, resulting in a reduction in bond strength (13), especially after soaking in body fluid (11, 17). Therefore, a suitable heat treatment is required that will offer control of the solubility of the CaP coating without weakening its adhesion to the Ti substrate.

It has been reported that rapid, homogeneous, and comparatively low-temperature heating at 600-700 °C such as defocused infrared radiation allows control of CaP solubility and ensures adherence of coatings for both ion-sputtering (IS) and IBDM (17, 18). In rapid-heating with infrared radiation at 400°C (Fig.8a), the coatings disappeared and many precipitates appeared on the Ti substrate after immersion in SBF. No apparent change was observed in the coatings rapidly heated at 600 °C due to limited dissolution of coatings (Fig.8b). However, many cracks were observed on the coatings rapidly heated at 800 °C (Fig.8c).

Fig.9 shows change in film thickness of IBDM-coatings relative to approximately 1.0 µm as-deposited coatings in SBF. The coatings on the as-deposited specimens (As) almost completely disappeared after 1 day. Furnace-heated coatings (HV) decreased in thickness depending on length of time of immersion, and the standard deviation is greater than the measured value after 5 weeks of immersion which may have been caused by partial peeling of the coatings. The coatings rapidly heated with infrared radiation at 400°C (IR400) almost disappeared after 1 week, whereas those of IR600 and IR800 retained approximately 60% and 55% of their thickness, respectively, after 5 weeks of immersion. This tendency is believed to be correlated with the crystallinity of the coatings. The IR800 specimens, however, showed large standard deviations after 1 week and 5 weeks of immersion due to partial peeling of coatings.

Table 1 shows change in tensile bond strength between coatings and Ti substrate after immersion in SBF (11, 17). The times until the maximum temperature was reached were approximately 8, 10, 13, and 26 seconds for IR400, IR500, IR600, and IR800, respectively.
Table 1 also shows change in surface morphology with dissolution or cracking of the coatings. As-deposited coatings and rapid-heated coatings at 400°C with infrared radiation dissolved within a couple of days. Cracks were observed in the coatings with furnace heating at 500°C for one hour and rapid heating at 800°C. Rapid heating at 600 and 700°C yielded high bond strength. These results indicate that high temperatures and long duration are unnecessary in obtaining crystallinity in thin coatings.

Dissolution of coated film and/or precipitation of calcium phosphate from the solution on the surface appear to occur simultaneously. With HAp, the SBF solution is supersaturated with Ca and P, so precipitation should be the major reaction if HAp is crystallized completely. However, dissolution is often observed in CaP coatings. Dissolution is dependent on the crystallinity, grain size, and density of CaP films. Low crystallinity, small grain size, lower density and the presence of impurities such as CO$_3^-$ give higher solubility (21, 22, 23, 24, 25).

2.3. Mechanism for debonding of CaP coatings

Reduction in bond strength is observed in heat-treated specimens. This appears to be a result of internal stresses caused by change in film density and the formation of titanium oxides and Ti-P compounds due to diffusion of elements. This exerts an adverse influence on the bond between the CaP coating and the Ti substrate (19).

The behavior of the elements involved in the debonding mechanism at the CaP coating/Ti interface after heat-treatment has been revealed (17). The results of XPS analyses of the interface between the coating and the Ti substrate (depth profile of Ti substrates, in which CaP coatings were removed by epoxy glue) are shown in Fig 10a. The intensities of the P$^3$ states (Ti$_3$P$_4$) of the furnace-heated and 800°C rapid-heated specimens were larger than those of
as-deposited or 600°C rapid-heated specimens, and the intensities of the Ca2p spectrum decreased in the furnace-heated and 800°C rapid-heated specimens. In addition, no remarkable differences in Ti-oxidation state (TiO₂) were observed among the specimens. These results suggest that too much growth of Ti-P compounds and decreased thickness of Ca-implanted layers is a major reason for cracks due to debonding of coatings. According to the binary phase diagram, the Ti-Ca system has neither solid solutions nor intermetallic compounds. In contrast, the Ti-P system has many intermetallic compounds such as Ti₃P₄, Ti₃P, Ti₅P₃ and Ti₂P. Therefore, if too much thermal energy is applied to CaP coated titanium, P diffuses toward the Ti substrate creating many Ti-P compounds at the interface, which results in cracks in the coatings due to debonding, and the Ca diffuses out of the Ti substrate (Fig.10b). Titanium oxide is not considered to play a dominant role in bonding between thin CaP coatings and Ti substrates.

2.4. Immobilization of bisphosphonates and simvastatin

Immobilization of osteogenesis-promoting drugs on thin CaP coatings is a promising new approach to achieve rapid osseointegration and improvement in the bone bed at the bone tissue/implant interface. In the treatment of osteoporosis, bisphosphonates and simvastatins have been reported to stimulate bone formation (26) and increase expression of BMP-2, respectively (27).

Bisphosphonates work not only as potent inhibitors of osteoclastic bone resorption but also exert a direct effect on osteoblasts (28). It was reported that bisphosphonates were able to immobilize on titanium implants through thin CaP coatings (29,30,31). The reason for this may be their marked affinity to calcium phosphates. XPS and FT-IR analyses revealed that titanium surfaces modified with thin CaP coatings allowed immobilization of bisphosphonate, and that
such surfaces revealed physiologically active functional groups of bisphosphonate origin (Fig.11). Alkaline phosphatase expression activity and bone-like nodule formation in rat bone marrow cells showed a greater increase on plates with immobilized bisphosphonate than on as-received titanium, indicating that bisphosphonate-immobilization has no toxic effect on osteoblasts, and that it provides a favorable micro-environment conducive to osteogenesis (29, 32). In an in vivo test using beagle dogs, fluorescence was widely observed in newly formed bone tissue around bisphosphonate-immobilized implants, and the ratio of bone contact to the bisphosphonate-immobilized implants was significantly higher than in other implants at twelve weeks(30). In addition, confocal laser scanning microscopy revealed that new bone widths significantly increased around bisphosphonate-immobilized implants compared with pure titanium implants (31) (Fig.12). These results suggest that thin CaP coatings on titanium surfaces enable immobilization of bisphosphonates, and that bisphosphonate-immobilized surfaces promote osteogenesis around medical implants. In addition, the concentration of released bisphosphonates can be controlled by regulating the crystallinity of HAp coatings as a carrier (33).

On the simvastatin (SV), since only the open-ring or beta-hydroxy acid forms (SVA) exhibit the efficacy of this medication for topical application, SV should be hydrolyzed to SVA. Accordingly, immobilization of SVA onto dental implants is expected to promote osteogenesis around dental implants. Thin-film of hexamethyldisiloxane {HMDSO, (CH₃)₃SiOSi(CH₃)₃} was plasma-polymerized onto titanium, then HMDSO surfaces were activated by O₂-plasma treatment (34, 35), resulting that hydroxyl group or O₂-functional groups were introduced to immobilize the SVA (Fig.13). Adsorption assay of SVA using a quartz crystal microbalance-dissipation (QCM-D) instrument demonstrated the largest amount of SVA was adsorbed on O₂-plasma treated HMDSO surfaces compared to untreated titanium,
HMDSO-coated titanium, and O$_2$-plasma treated titanium. These findings suggested that the adsorption of SVA was enhanced on more hydrophilic surfaces concomitant with the presence of an OH group and/or O$_2$-functional group resulting from the O$_2$-plasma treatment (Fig.14), and that an organic film of HMDSO followed by O$_2$-plasma treatment is a promising method for the adsorption of SVA in dental implant systems.

Controlled release of SVA by means of its topical application around dental and maxillofacial implants would promote osteogenesis in surrounding bone tissue. Because of their multi-functional characteristics and bioadaptability, cyclodextrins (CDs) are capable of forming inclusion complexes with many drugs by including a whole drug molecule inside their cavity (Fig.15). The SVA release properties from SVA/CD coatings with different pH values were evaluated as well as the characteristics of the coatings (36). The results showed that the number of SVA/CD complexes formed depended on the pH of the solution, and that subsequent release of SVA from the coatings depended on the number of complexes and resulting crystallinity of the coatings (Fig.16). These results suggest that SVA/CD complexes offer potential in bone generation with a drug delivery system around dental and maxillofacial implants.

3. Soft tissue / Implant interface

Dental implants lack the structures that maintain the continuity between the epithelium and connective tissues that are normally formed by hemidesmosomes and the basal lamina, which connect dental enamel and adhesive epithelium. Peri-implant epithelium has a reduced capacity to act as a proliferative defense mechanism than does the junctional epithelium (37, 38). Therefore, to prevent the invasion of the bacteria and epithelium, a system of biological sealing is required. We found that the extension and spread of fibroblasts and epithelial cells were critically influenced by the pore diameter of 1.2-3.0 µm in Millipore filters. Our observation of in vitro experiments also
suggests that a range in hole size of 50 to 100 µm is most critical for the connective tissue cells to migrate and orient at right angles to the implant surface, similar to Sharpey’s fibers. Multi-grooves, a combination macro-grooves and micro-grooves, are also considered to be useful for ECM production with a contact guidance (39) (Fig 17). These surface topographies may help in providing a biological seal around the implant.

As for the surface physico-chemistry, methods of modifying the titanium surface using adhesive proteins such as fibronectin or laminin-5 compatible with the soft tissue/implant interface have been proposed. For the implant surface in contact with subepithelial connective tissues, tresyl chloride treatment is used to adhere the selected proteins such as fibronectin to the amino residues (40). The gingival epithelium attached to dental implants through the formation of hemidesmosomes using laminin-5 (41). A stable coating and prevention of protein denaturation at the time of implantation are necessary.

4. Oral fluid / Implant interface

Microbial plaque accumulation surrounding dental implants may develop into peri-implantitis, which is defined as inflammation or infection around an implant, with accompanying bone loss. Biofilm accumulations are observed surrounding titanium implants, and many kinds of bacteria, which were confirmed to be the same as periodontopathic bacteria (42), are recognized in the biofilm formation (Fig. 18).

It is therefore important to maintain the surface of dental implants exposed to the oral cavity (Oral fluid / Implant interface) free of biofilm to prevent peri-implantitis. There are at least two methods of inhibiting the formation of microbial plaque. The first is to inhibit the initial adhesion of oral bacteria. The second is to inhibit the colonization of oral bacteria, which involves surface antimicrobial activity. The adhesion of bacteria is greatly influenced by electric
charges on the implant surface because bacteria have a large specific surface area. Antimicrobial modification can be effective for the implant surface. Another requirement for the modified surfaces is their resistance to wear when the teeth are brushed.

4.1 Initial adhesion of oral bacteria

The initial adherence of oral bacteria on cp-titanium and titanium surfaces modified with a cold-plasma was investigated (43) (Table 2). Surface modifications were conducted with cold plasmas that included ion implantation (Ca\(^+\), N\(^+\), F\(^+\)), oxidation (titania spraying), ion plating (TiN, alumina), and ion beam mixing (Ag, Sn, Zn, Pt) with Ar\(^+\) on polished pure titanium plates. The results showed that comparatively large amounts of \textit{P. gingivalis} and \textit{A. actinomycetemcomitans}, which are major periodontopathic bacteria, adhered to polished cp-titanium. These findings indicate that there is a probable risk of bacterial adhesion to titanium surfaces at the supra- and sub-gingival portions of implants, and surface modification to inhibit the adherence of oral bacteria is required. The degree of \textit{P. gingivalis} adhesion showed a positive correlation with surface energy and the amount of calcium-ion adsorption.

The level of bacterial adhesion on calcium-implanted surfaces was greater than on polished titanium, despite similar degrees of surface roughness. The reason for this is believed to be that the Ca-rich surfaces on the calcium-implanted specimens promoted protein adsorption in saliva and, ultimately, bacterial adhesion. Accordingly, even though calcium-ion implantation is beneficial in bonding implants to bone tissue, this treatment carries with it the risk of promoting the adhesion of biofilm on surfaces exposed to the oral cavity. In contrast, the level of initial adhesion of bacteria decreased on the alumina-coated specimen. This is related to the nonadsorption of calcium ions on alumina-coated specimens. In contrast to titanium oxide, the isoelectric point of \(\alpha\text{-Al}_2\text{O}_3\) is reported to be 9.2 and that of \(\gamma\text{-Al}_2\text{O}_3\) is reported to be 8.0.
Therefore, the surface of the alumina-coated specimen is considered to be positively charged, and calcium ions were not adsorbed on the surface, resulting in a decrease in the initial levels of adhered *P. gingivalis*.

### 4.2 Antimicrobial activity

Antimicrobial activity was also investigated on the same specimens as the initial adhesion assay (44). F⁺-implanted specimens significantly inhibited the growth of both *P. gingivalis* and *A. actinomyctemcomitans* (Fig.19). Fluoride is widely used as a highly effective anticaries agent. The principal antibacterial mechanism considered was that a metal fluoride complex affects bacterial metabolism as an enzyme inhibitor. Incidentally, it was confirmed that F⁺-implanted surfaces did not influence the proliferation of mouse-fibroblast cells. Titania-sprayed specimens generated no antimicrobial activity despite the anatase that formed on the surfaces. This may be because no UV light was used, and no coupling metals were used for stimulating photocatalytic reactions.

A monoclonal anti-human cystatin-SA (cysteine protease inhibitor) antibody for cystatin-SA and a histatin5, antimicrobial peptides, were immobilized onto those titanium surfaces. The amounts of adsorbed anti-cystatin and histatin5 were increased by O₂ plasma surface modification using a quartz crystal microbalance (QCM-D) technique, and the amount of *C. albicans* colonization on histatin5-adsorbed specimens was significantly less than the control (45) (Fig.20). There is no significant difference in the amount of initial attachment of *C. albicans* among the control (PMMA), O₂-treated PMMA and the histatin5-adsorbed PMMA. However, the amount of *C. albicans* biofilm formation on the histatin5-adsorbed PMMA significantly decreased compared to that on the other specimens. These results indicate that histatin5-adsorption does not prevent or reduce adhesion of the microorganism to the denture.
surface, but that direct candidicidal activity of the adsorbed molecules is responsible for reducing *C. albicans* biofilm formation on the denture surface.

Modification of titanium surfaces with conjugated molecules consisting of antimicrobial and hexapeptidic titanium-binding peptides (minTBP-1) is also useful. (46) Titanium-binding peptide (minTBP-1) was originally isolated as a 12-mer peptide, and mutational analyses revealed that the N-terminal hexapeptide RKLPDA (named minTBP-1) was sufficient for binding (Fig.21) (47, 48). Mutational studies also indicated that the first arginine (R1), fourth proline (P4) and fifth aspartate (D5) were important for binding. Because the surface of titanium is covered with an oxide film displaying both positively- and negatively-charged hydroxyl groups under physiological conditions, electrostatic interactions between –O\(^-\) and R1, and –OH\(^2+\) and D5, have been proposed to underlie the interaction between minTBP-1 and titanium.

Four kinds of peptide were prepared as summarized in Table 3. Adsorption assay of the synthesized peptides was carried out on crystal quartz sensors coated with Ti using a QCM-D instrument. The results demonstrated that accretion of surfactant reduced nonspecific interactions, dramatically enhancing the selectivity and specificity of the Ti-binding peptides, and ensuring reversible specific binding (Fig.22). In addition, the bioactivities of *P. gingivalis* cells on peptide-modified titanium were evaluated by ATP-bioluminescent assay. The bioactivity test revealed that ATP activity in *P. gingivalis* in peptide-modified specimens significantly decreased compared to that in the Ti control. These findings indicate that surface modification with conjugated molecules consisting of antimicrobial and titanium-binding peptides is a promising method for reduction of biofilm formation on titanium implants.

In conclusion, a cold-plasma surface modification is useful in controlling the physicochemical nature of surfaces, including the surface energy and the surface electrical charge, leading to
immobilize the drugs and peptides, and in developing bio-functional implants (Fig.4). Considering the present technology, it may be possible to produce implants with highly controlled surfaces that maintain homeostasis.

**Acknowledgements**

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**Conflicts of Interest**

The authors have no financial relationship with the organization that sponsored the research.

**REFERENCES**


Figure legends

Fig. 1  Isoelectric point (x-axis) and hydropathy index (y-axis) of amino acids

Fig. 2  Schematics of surface modification using a cold-plasma

Fig. 3  Relationship between film thickness and energy of atoms, which is related to adherence of coatings.

Fig. 4  Bio-functionalization of dental implants with surface modification

Fig. 5  CaP coatings produced by plasma spraying method: Scanning electron micrograph (SEM) of cross section (a), and optical micrograph of surface after soaking in 0.9% NaCl solution at pH 6.0 for 30 weeks (b).

Fig. 6  (a) Brånemark implant (left) and CaP coated implant by IBDM method; (b) SEM images of cross section of CaP coatings produced by IBDM; (c) SEM images of IP coatings after bending test; lower image in b and c show higher magnification of area shown in rectangle in upper micrograph.

Fig. 7  Thin XRD profiles of as-deposited (a), furnace-heated at 500 ºC for 4 hrs (b), rapid heating with infrared radiation at 500 ºC (c) and 700 ºC (d), and HAp powder as evaporant for coating (e).

Fig. 8  SEM of coatings by rapid heating with infrared radiation at 400ºC (a), 600ºC (b), and 800ºC (c) after immersion in SBF for 35 days. Lower images in a, b, c show higher magnification of area shown in rectangle in upper micrograph.

Fig. 9  Change in film thickness of coatings relative to as-deposited thickness of approximately 1.0 µm in SBF.

Fig. 10  (a) XPS depth profiles of Ti substrates in which Ca-P coatings were removed by epoxy glue, (b) diffusion of P and Ca, and creation of many Ti-P compounds at the interface that leads to debonding of CaP coating if too much thermal energy is applied to CaP-coated titanium

Fig. 11  Possible structure of immobilization of bisphosphonates (Pamidronate disodium) to titanium through thin Ca-P coatings.

Fig. 12  Confocal laser scanning microscopy (upper, Cal: calcein (1w), Alc: alizarin complexone (3 wks) and new bone width of Ti and Bisphosphonate-immobilized implants in wistar rats (lower)  Imp: implant, NB: new bone

Fig. 13  XPS spectra of Ti, Ti+O2, HMDSO, and HMDSO+O2 specimens

(HMDSO: hexamethyldisiloxane, O2: O2-plasma treatment)

Fig. 14  Possible structure change of HMDSO monomers (a) by plasma polymerization (b), following O2-plasma treatment (c), and immobilization mechanism of SVA (d).

Fig. 15  Chemical structure (upper) and toroidal shape (lower) of β-cyclodextrin molecule.
Fig. 16 Release profiles of SVA from films coated onto titanium substrates using SVA/CD solutions with different pHs.

Fig. 17 Multi-grooves, a combination of macro-grooves (approximately 50µm) and micro-grooves (approximately 1µm), to control the orientation of both cells and extracellular matrix (ECM) such as collagen.

Fig. 18 X-ray photograph around peri-implantitis (black arrows, upper) and microbial plaque accumulation surrounding the abutment of dental implants (lower)

Fig. 19 Antimicrobial activity of *P. gingivalis* on surface-modified titanium for 48 h.

Fig. 20 Shift in frequency against time for exposure of the specimen to *C. albicans*. (a) Immobilized with histatin-5, (b) control. The SEM images are also shown in Fig. 25.

Fig. 21 Schematics of titanium binding peptide (min TBP-1).

Fig. 22 Shift in frequency (∆f) against time for exposure of Ti sensor to various peptides obtained by QCM-D as a typical example. At black arrowhead, 100µg mL⁻¹ peptide in PBS-T was injected, followed by successive injection of PBS-T (white arrowhead). Decrease in frequency shows mass adsorption on the surfaces. All experiments except “Lysozyme w/o blocking” were carried out with the surfactant, i.e. with blocking.
Table 1 Change in bond strength (MPa) between coatings and Ti substrate after immersion in SBF for 35 days. Tensile bond strength was measured in specimens showing no change in surface morphology.

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<tr>
<td>As-deposited</td>
<td>59.0(10.5)</td>
<td>Dissolved</td>
</tr>
<tr>
<td>Furnace heated at 500ºC for 1 h</td>
<td>46.3(17.0)</td>
<td>Cracked</td>
</tr>
<tr>
<td>Infrared radiation (rapid heating)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400 ºC</td>
<td>59.6(13.0)*</td>
<td>Dissolved</td>
</tr>
<tr>
<td>500 ºC</td>
<td>55.7(6.9)*</td>
<td>Peeled partially</td>
</tr>
<tr>
<td>600 ºC</td>
<td>60.2(13.8)*</td>
<td>44.9(9.6)</td>
</tr>
<tr>
<td>700 ºC</td>
<td>57.8(9.6)*</td>
<td>48.4(11.7)</td>
</tr>
<tr>
<td>800 ºC</td>
<td>33.5(15.4)</td>
<td>Cracked</td>
</tr>
<tr>
<td>Modification/Treatment</td>
<td>Characterization</td>
<td>Thickness</td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Ti-polished</td>
<td>TiO₂</td>
<td>&lt;30 nm</td>
</tr>
<tr>
<td>HAₚ-coated</td>
<td>IBDM Hydroxyapatite</td>
<td>1 µm</td>
</tr>
<tr>
<td>Ca-implanted</td>
<td>CaTiO₃, TiO₂, TiO</td>
<td>150 nm</td>
</tr>
<tr>
<td>N-implanted</td>
<td>Ion implantation TiN, Ti₂N, TiO₂</td>
<td>300 nm</td>
</tr>
<tr>
<td>F-implanted</td>
<td>TiF₃, TiOF, TiO₂, TiO</td>
<td>150 nm</td>
</tr>
<tr>
<td>Anode-oxidized</td>
<td>TiO₂ (brookite), TiO</td>
<td>300 nm</td>
</tr>
<tr>
<td>Titania-sprayed</td>
<td>Frame spraying TiO₂ (rutile, anatase)</td>
<td>&gt;3 µm</td>
</tr>
<tr>
<td>TiN-coated</td>
<td>Ion plating TiN</td>
<td>3 µm</td>
</tr>
<tr>
<td>Alumina-coated</td>
<td>Al₂O₃ (corundum)</td>
<td>3 µm</td>
</tr>
<tr>
<td>Ag-IBM</td>
<td>Ag, TiO₂, TiOx</td>
<td>100 nm</td>
</tr>
<tr>
<td>Sn-IBM</td>
<td>Ion bema mixing Sn, TiO₂, TiOx</td>
<td>150 nm</td>
</tr>
<tr>
<td>Zn-IBM</td>
<td>Zn, TiO₂, TiOx</td>
<td>100 nm</td>
</tr>
<tr>
<td>Pt-IBM</td>
<td>Pt, TiO₂</td>
<td>150 nm</td>
</tr>
<tr>
<td>HMDSO</td>
<td>Plasma polymerization CH₃, SiO₄</td>
<td>100nm</td>
</tr>
<tr>
<td>O₂-plasma</td>
<td>Plasma surface treatment C-OH, COOH</td>
<td>10nm</td>
</tr>
<tr>
<td>N₂-plasma</td>
<td>C-OH, NH₂</td>
<td>10nm</td>
</tr>
</tbody>
</table>

IBDM: Ion beam dynamic mixing, HMDSO: hexamethyldisiloxane, *wet process
Table 3. Antimicrobial peptides and conjugated peptides with Ti-binding peptide (minTBP-1).

<table>
<thead>
<tr>
<th>Classification</th>
<th>Amino acid sequence (single letter code)</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histatin5</td>
<td>DSHAKRHHGYKRKFHEKHHSHRGY</td>
<td>His5</td>
</tr>
<tr>
<td>minTBP-1 + Histatin5</td>
<td>RKLPDAPDSHAKRHHGYKRKFHEKHHSHRGY</td>
<td>minTBP1+His5</td>
</tr>
<tr>
<td>Lactoferricin</td>
<td>FQWQRNMRKVR</td>
<td>Lfcin</td>
</tr>
<tr>
<td>minTBP-1 + Lactoferricin</td>
<td>RKLPDAPGGFQWQRNMRKVR</td>
<td>minTBP1+ Lfcin</td>
</tr>
</tbody>
</table>
Fig. 1 Isoelectric point (x-axis) and hydropathy index (y-axis) of amino acids.
Fig. 2  Schematics of surface modification using a cold-plasma
Fig. 3 Relationship between film thickness and energy of atoms, which is related to adherence of coatings.
Fig. 4 Bio-functionalization of dental implants with surface modification

**Oral fluid / Implant interface**
- No biofilm formation
- F⁻-implantation
- Antimicrobial peptides

**Soft tissue / Implant interface**
- Easily form hemidesmosomes
  - Laminin-5 recognition peptide
- Adhere to the subepithelial connective tissues
  - Multi-grooves + fibronectin, collagen

**Bone tissue / Implant interface**
- Early osseointegration
- Improve the host bone bed
  - Thin calcium phosphate coating
  - Osteogenesis-promoting drug
    - (bisphosphonate, simvastatin)
Fig. 5 CaP coatings produced by plasma spraying method: Scanning electron micrograph (SEM) of cross section (a), and optical micrograph of surface after soaking in 0.9% NaCl solution at pH 6.0 for 30 weeks (b).
Fig. 6  (a) Brånemark implant (left) and CaP coated implant by IBDM method; (b) SEM images of cross section of CaP coatings produced by IBDM; (c) SEM images of IP coatings after bending test; lower image in b and c show higher magnification of area shown in rectangle in upper micrograph.
Fig. 7 Thin XRD profiles of as-deposited (a), furnace-heated at 500 °C for 4 hrs (b), rapid heating with infrared radiation at 500 °C (c) and 700 °C (d), and HAp powder as evaporant for coating (e).
Fig. 8 SEM of coatings by rapid heating with infrared radiation at 400°C (a), 600°C (b), and 800°C (c) after immersion in SBF for 35 days. Lower images in a, b, c show higher magnification of area shown in rectangle in upper micrograph.
Fig. 9 Change in film thickness of coatings relative to as-deposited thickness of approximately 1.0 µm in SBF.
Fig. 10 (a) XPS depth profiles of Ti substrates in which Ca-P coatings were removed by epoxy glue, (b) diffusion of P and Ca, and creation of many Ti-P compounds at the interface that leads to debonding of CaP coating if too much thermal energy is applied to CaP-coated titanium.
Fig. 11 Possible structure of immobilization of bisphosphonates (Pamidronate disodium) to titanium through thin Ca-P coatings.
Fig. 12 Confocal laser scanning microscopy (upper, Cal: calcein (1w), Alc: alizarin complexone (3 wks) and new bone width of Ti and Bisphosphonate-immobilized implants in wistar rats (lower)  Imp: implant, NB: new bone
Fig. 13 XPS spectra of Ti, Ti+O2, HMDSO, and HMDSO+O2 specimens (HMDSO: hexamethyldisiloxane, O2: O₂-plasma treatment)
Fig. 14 Possible structure change of HMDSO monomers (a) by plasma polymerization (b), following O$_2$-plasma treatment (c), and immobilization mechanism of SVA (d).
Fig. 15 Chemical structure (upper) and toroidal shape (lower) of β-cyclodextrin molecule.
Fig. 16 Release profiles of SVA from films coated onto titanium substrates using SVA/CD solutions with different pHs.
Fig. 17 Multi-grooves, a combination of macro-grooves (approximately 50µm) and micro-grooves (approximately 1µm), to control the orientation of both cells and extracellular matrix (ECM) such as collagen.
Fig. 18 X-ray photograph around peri-implantitis (black arrows, upper) and microbial plaque accumulation surrounding the abutment of dental implants (lower)
Fig. 19  Antimicrobial activity of *P. gingivalis* on surface-modified titanium for 48 h.
Fig. 20  Shift in frequency against time for exposure of the specimen to *C. albicans*. (a) Immobilized with histatin-5, (b) control. The SEM images are also shown in Fig. 20.
Fig. 21  Schematics of titanium binding peptide (min TBP-1).
Fig 22. Shift in frequency (Δf) against time for exposure of Ti sensor to various peptides obtained by QCM-D as a typical example. At black arrowhead, 100µg mL⁻¹ peptide in PBS-T was injected, followed by successive injection of PBS-T (white arrowhead). Decrease in frequency shows mass adsorption on the surfaces. All experiments except “Lysozyme w/o blocking” were carried out with the surfactant, i.e. with blocking.