Pilot study of gingival connective tissue responses to 3-dimensional collagen nanofiber-coated dental implants

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The aim of the present study was to evaluate the gingival connective tissue response to screw-type titanium implants coated with Type I collagen nanofibers, which were prepared using the electrospray deposition method. Implants were immediately inserted into the socket of maxillary first molars after the extraction. Undecalcified sections after 4 weeks implantation were histologically observed. Better contact of the gingival connective tissue was generally observed around the collagen nanofiber-coated implants than titanium and non-fibrous collagen-immobilized implants. Gingival connective tissue to implant contact was significantly greater with the collagen nanofiber-coated implants than with the titanium and collagen-immobilized implants at the distal side, but not at the mesial side. Polarized light microscopy revealed that some birefringent collagen fiber bundles are oriented perpendicularly to the implant surfaces in the gingival connective tissue adjacent to the collagen nanofiber-coated implants. Collagen nanofiber-coating may have a possibility for improving gingival connective tissue response to titanium implants.

Keywords: Collagen nanofiber, Electrospray deposition, Titanium, Gingival connective tissue attachment

INTRODUCTION

The tight bonding of titanium dental implants to bone is known as osseointegration and various kinds of surface modifications, such as blasting or calcium phosphate coating, have been reported to improve the bone response to titanium implants. In contrast, fewer numbers of studies have been reported for the surface modification of titanium regarding the attachment of soft tissue such as the gingiva to titanium implants. Some studies reported that soft and hard tissue integration was mainly influenced by surface hydrophilicity of the dental implant surface. Kloss et al. demonstrated that the hydrophilicity influenced connective tissue healing and the inflammatory response was decreased at the hydrophilic surface after the implantation of hydrophilic nano-crystalline diamond-coated titanium into the subcutaneous connective tissue of rats. The effectiveness of mechanical treatments for gingival connective tissue attachment has also been investigated. It is reported that laser-ablated micro-grooved surfaces allowed for direct gingival connective tissue attachment to titanium implants and prevented epithelial downgrowth. The efficacy of mesh structures for the mechanical attachment of soft tissue to implants has also been demonstrated. Nylon or titanium mesh-coated titanium implants effectively attained an anchor in soft tissue following subcutaneous implantation in rats.

The fiber structures of collagens mainly consisted in gingival tissue and cementum around natural teeth. Thus, coating titanium surfaces with collagen may enhance cell activity or initial cell attachment around titanium implants, resulting in improved tissue responses. Nagai et al. reported that collagen coating enhanced the attachment of the human gingival fibroblasts in comparison with non-coating. Morikawa et al. immobilized collagen on titanium surface using gold deposition and production of a stable monomolecular layer with cysteine. They implanted the collagen immobilized implants into the mandibular of beagle dogs and found that collagen immobilization onto titanium improved the adhesion against gingival tissue. Thus, it is presumed that the collagen immobilization or coating will be one useful method for improving the attachment of gingival connective tissue to titanium implants.

Several methods have been reported for immobilizing cell-adhesive proteins such as collagen to titanium surface. Hayakawa et al. recently developed a convenient method, the tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride)-activated technique, for directly immobilizing cell-adhesive proteins onto a titanium surface. Previous studies demonstrated that the immobilization of cell-adhesive proteins such as fibronectin or collagen onto titanium using the tresyl chloride method enhanced cell attachment, provided a wider range of morphologies of attached cells, and altered the gene expression profile. Yoshida et al. reported that immobilized fibronectin on titanium influenced the arrangement of the attached osteoblast-like cells.

On the contrary, it is postulated that 3-dimensional fiber networks are suitable for enhancing cell activities such as proliferation and/or differentiation. It is also expected that formation of 3-dimensional mesh-like...
structure of collagen fibers onto titanium might allow gingival collagen fiber penetrate into fiber structure and provide tight attachment of gingival connective tissue to titanium implant.

Electrospray deposition (ESD) method can produce collagen and/or collagen/apatite and collagen/poly(glycolic acid) nanofibrous scaffolds as a biomimetic 3-dimensional nanofibrous extracellular matrix for tissue engineering\textsuperscript{20-22}. ESD is a process through which a liquid is transformed into a fine mist by the application of a high-voltage electric field to the capillary or syringe. The principle of ESD is illustrated in Fig. 1. Cell growth, the penetration of cells into the fiber matrix, and the enhancement of cell attachment and proliferation has been reported in electrospun collagen nanofibrous scaffolds\textsuperscript{22}. Electrospun scaffolds consisting of polycaprolactone, collagen, and nanoparticulate hydroxyapatite were reported to support the adhesion, integrin-related signaling, and proliferation of mesenchymal stem cells, while the pore size of electrospun scaffolds influenced cell infiltration\textsuperscript{23}. Hayakawa \textit{et al.}\textsuperscript{24} confirmed the formation of collagen nanofibers on titanium disk surfaces by changing some conditions for ESD, such as the collagen concentration, flow rate and voltage applied.

In the present study, we attempted to deposit collagen nanofibers on screw-type titanium implants using the ESD method and subsequently evaluate the gingival connective tissue response to titanium implants covered with collagen nanofibers following implantation into the sockets of the rat maxillary first molars. The gingival connective tissue response was also evaluated in collagen-immobilized titanium, which was produced using the tresyl chloride-activation technique. In this case, immobilized collagen does not have 3-dimensional fiber structure. We hypothesized that 3-dimensional collagen nanofibers will produce better gingival connective tissue attachment to titanium.

![Fig. 1 Schematic drawing of the principle underlying electrospray deposition.](image1.png)

**MATERIALS AND METHODS**

**Materials**

Casted screw-type titanium (Ti) implants (Ti, 99.8 mass%, APEX, Tokyo, Japan), measuring 1.5 mm in diameter and 3.0 mm in length, were used in the present study. The diameter and length of the titanium screw implants were set after comparing them with those of the extracted rat tooth, as shown in Fig. 2.

**Immobilization of collagen with the tresyl chloride-activated technique**

Ti implants were ultrasonically cleaned with ethanol prior to protein immobilization. The surfaces of Ti implants were then completely covered with tresyl chloride (2,2,2-trifluoroethanesulfonil chloride, Fluka, Buchs, Switzerland) and coated specimens were stored at 37ºC for 2 days. Tresylated Ti implants were rinsed with double-distilled water, dried, and stored in a desiccator.

Collagen-immobilized Ti (Coll) implants were prepared as follows. Type I collagen (AteloCell, IPC-50, Koken, Tokyo, Japan) was dissolved in phosphate-buffered saline (PBS) solution, pH 7.4, at a concentration of 100 µg/mL. Tresylated Ti implants were immersed in collagen/PBS solution for 24 h at 37ºC and then rinsed with doubled-distilled water. Implants were then dried with a gentle stream of dry air.

**Electrospray deposition (ESD) of collagen nanofibers**

ESD was performed using an ESD device (ES-2000, Fuence, Wako-shi, Japan). Collagen nanofibers were sprayed onto the screw-type Coll implants, not the Ti implants because of enhanced bonding of collagen nanofibers to the implant material. The base plate of
the apparatus was covered with aluminum foil. Screw-type Coll implants were connected to a stainless tube and lifted to cover the whole surface of these implants. Type I collagen (Atelocollagen powder, Koken) was dissolved in 1,1,1,2,2,2-hexafluoro-2-propanol (HFIP, Wako, Osaka, Japan) at a concentration of 50 mg/mL. The collagen HFIP solution was sprayed from a glass syringe. The distance between the glass syringe and substrate surface was 10 cm. The voltage applied between the glass syringe and substrate was 16 kV, and the flow rate of the collagen HFIP solution was 5 µL/min. The collagen HFIP solution was electrosprayed for 10 min. Nanofiber spraying was performed in an acrylic chamber under atmospheric conditions. Thus, electrospun collagen nanofiber-coated (Coll-nanofiber) implants were obtained. The deposited nanofibers were fixed with glutaraldehyde and coated with gold, and the morphologies of nanofibers were then observed using a scanning electron microscope (SEM, JSM-5600LV, JEOL, Tokyo, Japan) at an accelerating voltage of 15 kV.

Experimental design and implantation procedure
Before surgery, all Ti, Coll, and Coll-nanofiber implants were sterilized using ethylene oxide gas. Animal experiments were reviewed and approved by the Institutional Animal Care Committee of Tsurumi University School of Dental Medicine (Certificate Number: 24A039). A total of twelve male Wistar rats with a weight of approximately 180 g (6 week old) were used. The rats were housed two per cage at 20–25°C in a 12-h circadian light rhythm and fed a powdered diet and tap water ad libitum during the experimental period.

Each animal received one implant. A total of twelve implants were inserted for 4 weeks; four Ti, four Coll, and four Coll-nanofiber implants.

Surgical interventions were conducted under general anesthesia by an intraperitoneal injection of ketamine hydrochloride (47 mg/kg) and medetomidine hydrochloride (0.4 mg/kg). The maxillary first molar on the right side was extracted using forceps. After an incision was performed in the periodontal tissue, the sockets of the mesial roots of the molar on the right side were enlarged with a dental reamer (#90–140). The implant was fixed into the prepared root socket using a screwdriver, as shown in Fig. 3. Incisions in the periodontal tissue were closed with 7-0 polyamide non-resorbable sutures (BioFit-D, Washiesu, Tokyo, Japan). After the operation, rats were injected subcutaneously with benzyl penicillin G procaine (3,000,000 U/kg) and were awakened with an intraperitoneal injection of atipamezole hydrochloride (0.83 mg/kg). The sutures were removed 2 days after surgery.

Rats were euthanized 4 weeks following implantation by an overdose of diethyl ether, and the maxillae were then harvested. Each implant site, including the implant and soft and hard peri-implant tissues, was dissected using a diamond saw (Cutting Grinding System, BS-300CP band system, EXAKT, Apparatebau, KG, Norderstedt, Germany).

Specimens including the maxilla were fixed in 10% neutral buffered formalin for 7 days. Specimens were then dehydrated through a graded series of ethanol: 70%, 80%, 90%, 96%, and 100%, and embedded in methylmethacrylate (MMA) resin. After polymerization, non-decalcified sections were prepared using a cutting-grinding technique (EXAKT-Cutting Grinding System, BS-300Cp band system and 400 CS micro grinding system, EXAKT). Sections of approximately 50–70 µm in thickness were obtained.

Histological preparation and evaluation
The central, most ground section of each implant was chosen for morphological and morphometric assessments. Undecalcified sections were stained with methylene blue and basic fuchsine.

The interface between the gingival connective tissue and the implant was observed using light microscopy (BX51, Olympus, Tokyo, Japan, magnification 200×). For Coll-nanofiber implant, birefringent collagen fiber bundles and their orientations in the gingival connective tissue around the implant were observed by polarized light microscopy.

The ratio of gingival connective tissue contact to the implant was defined as the percentage of direct gingival connective tissue contact to the total perimeter of the screw implant located in the gingival connective tissue area over the alveolar crest around the implant. Measurements were carried out on the medial and distal sides using the image analysis system (WinROOF, Visual System Division, Mitani, Tokyo, Japan).

Statistical analysis
Quantitative data were statistically analyzed across the
three implant groups using a one-way analysis of variance (ANOVA) with Graph Pad Prism 5.0 c (GraphPad Software, San Diego, CA, USA). Bonferroni corrections were performed to probe pairwise comparisons. *p* Values of less than 0.05 were considered significant and data were expressed as the mean ± standard deviation (SD).

**RESULTS**

Figure 4 shows the SEM images of collagen nanofibers sprayed on titanium. The deposition of uniform collagen nanofibers with a diameter of approximately 200–500 nm was observed.

Animals remained in good health throughout the animal experiments. No clinical signs of inflammation or adverse tissue reactions were noted when animals were sacrificed, and loosening of the implants was not observed.

The histological appearances of the gingival connective tissue that formed over the alveolar crest around the implants are shown in Fig. 5. The gingival connective tissue responses induced by the different implants were clearly different. Better contact between the gingival connective tissue and the implant was generally observed with the Coll-nanofiber implants than with the Ti and Coll implants.

We then observed the undecalcified specimens under crossed polaroids with gypsum plate (a first-order wave-plate) to observe the attachment and orientation of collagen fiber bundles adjacent to the Ti, Coll and Coll-nanofiber implant surfaces (Figs 6–8). The microscopic stage was rotated from 0–90 degree to observe all birefringent fibers with different orientations. As the retardation of the system will simply be the sum or subtraction of the retardations of the plate (550 nm) and the fibers, birefringent areas are colored either yellow or blue, depending on the local orientation of the collagen fibers relative to the polarizer-analyzer axis.

Polarized light microscopic images of birefringent collagen fiber bundles of the gingival connective tissue formed around the Ti, Coll and Coll-nanofiber implant are shown in Figs. 6–8. Figure 7 shows the magnified images of polarized light microscopic appearance of gingival connective tissue contacts to each implant in Figure 5. SEM images of collagen nanofibers sprayed onto screw-type titanium implants using the ESD technique.

Fig. 4  SEM images of collagen nanofibers sprayed onto screw-type titanium implants using the ESD technique.

Fig. 5  Histological appearance of gingival connective tissue contacts to the implants under transmitted light microscopy. a) Ti implant. Clear contact between gingival connective tissue and Ti implants was not observed at both the mesial and distal sides. b) Coll implant. Less contact was observed at the mesial side than at the distal side. c) Coll-nanofiber implant. Direct contact between gingival connective tissue and the implants was observed at the mesial and distal sides.
Fig. 6  Polarized light microscopic images of gingival connective tissue contacts to the implants. 
   a) Ti implant, No clear contact between gingival connective tissue and Ti implants. b) Coll implant, Less contact 
   between gingival connective tissue and the implants at the mesial side. c) Coll-nanofiber implant, Direct contact 
   between gingival connective tissue and the implants at the mesial and distal sides.

Fig. 7  Magnified images of polarized light microscopic appearance of gingival connective tissue contacts to the Ti (a), Coll 
   (b), and Coll-nanofiber (c) implants in the squared regions of Fig. 6. 
   Direct contact between gingival connective tissue and the implants was observed at the mesial and distal sides in the 
   Coll-nanofiber implants (arrows), but not in the other implants (asterisks).

Fig. 8  Higher magnifications of gingival connective tissue formed around Ti, Coll and Coll-nanofiber implants (the squared 
   regions of Fig. 7). 
   Specimens were observed under crossed polars without a first order wave plate. a) Ti implant, b) Coll implant, c) 
   Coll-nanofiber implant, and d) natural teeth (rat maxillary 2nd molar). No apparent contacts were seen between 
   the gingival connective tissues and Ti and Coll implants (asterisks). Some perpendicular birefringent collagen fiber 
   bundles are seen adjacent to the Coll-nanofiber implant surface (small white arrows) similarly to the fiber bundles 
   seen in the natural teeth (d). Some sectioned surfaces of the fiber bundles (surrounded by dotted lines) are also 
   seen.
the squared regions of Fig. 6. And, Fig. 8 shows higher magnifications of gingival connective tissue formed around each implant in the the squared regions of Fig. 7. Polarized light image of natural teeth was also shown in Fig. 8. The birefringent collagen fiber bundles were seen around the three implants (Figs 7 and 8). However, no apparent contacts by perpendicularly oriented collagen fibers were seen in the Ti and Coll implants (as indicated by asterisks; Figs 7 and 8). Some perpendicularly oriented birefringent collagen fibers were distinctly seen in the region adjacent to the Coll-nanofiber implant surfaces (as indicated by small white arrows; Figs 7 and 8) as seen in natural tooth (Fig. 8d).

Some sectional surfaces of the birefringent collagen fiber bundles are also seen adjacent to the Coll-nanofiber implant surfaces (surrounded by dotted lines; Fig. 8c).

Table 1 lists the percentages of the gingival connective tissue attachment around the three different implants. The gingival connective tissue attachment at the distal side was significantly greater with the Coll-nanofiber implants than with the Ti and Coll implants ($p<0.05$). At the mesial side, no significant differences were observed in the percentage of the gingival connective tissue attachment between the three different implant groups ($p>0.05$). No significant difference was observed at the distal side between the Ti and Coll implants ($p>0.05$). When both sides were considered, the mean values of the gingival connective tissue attachment ratio at the mesial and distal sides were significantly higher with the Coll-nanofiber implants than with the Ti implants ($p<0.05$), but was not greater than that with the Coll implants ($p>0.05$).

### DISCUSSION

In the present study, we deposited collagen nanofibers on screw-type titanium implants using the ESD method and evaluated gingival connective tissue attachment following implantation into the tooth sockets at the rat maxillary molars. The implants were inserted into the mesial root of the empty sockets according to the method by Raita et al. The ESD method has also been applied for surface modifications to titanium implants in addition to the preparation of scaffolds for tissue engineering. Schouten et al. deposited alkali phosphatase (ALP), calcium phosphate, or a combination of ALP and calcium phosphate coatings onto titanium and evaluated in vivo bone responses to the different coatings. In their study, the deposited ALP was a 2-dimensional film structure, not a 3-dimensional fiber structure. Our previous studies demonstrated that a collagen concentration of 50 mg/mL, flow rate of 5.0 mL/min, and applied voltage of 25 kV produced 3-dimensional uniform structures for collagen nanofibers with a diameter of approximately 200–280 nm. Thus, the same conditions were used to deposit collagen nanofibers onto the screw-type titanium implants. Screw-type titanium implants were held by a stainless tube for spraying such that collagen nanofibers could be sprayed onto the whole surface.

Gingival connective tissue responses were also examined in collagen-immobilized surfaces. Immobilized collagen by the tresyl chloride-activation method did not have a 3-dimensional structure. The efficacies of the 2-dimensional collagen film and 3-dimensional collagen nanofiber structure in improving gingival connective tissue attachment were compared. The results obtained revealed gingival connective tissue attachment at the distal side was better with the Coll-nanofiber implants than with the Ti and Coll implants, while the Coll implants were not effective for improving gingival connective tissue attachment. Therefore, it is suggested that the 3-dimensional collagen nanofiber structure was more effective for gingival connective tissue attachment than the 2-dimensional immobilized collagen film. This may have been due to the progressive penetration of gingival connective tissue into the pores of the collagen nanofibers, although the detailed mechanism should be clear further. It is postulated that 3-dimensional structure of collagen nanofibers may induce the elongation of connective tissue around the implants.

It also revealed that 3-dimensional collagen nanofiber could control the orientation of collagen fibers in the gingival connective tissue around the implants. Some perpendicularly oriented collagen fibers were present adjacent to the Coll-nanofiber implant surfaces. However, the amounts of the perpendicularly oriented collagen fibers were less than those in natural teeth. Further study should be needed to clear the mechanism for inducing the orientation of collagen fibers in gingival connective tissue around the Coll-nanofiber implants and more amounts of perpendicularly oriented collagen fibrils should be induced.

### Table 1 Percentage of gingival connective tissue attachment

| Implant          | Mesial side | Distal side | Both side |
|------------------|-------------|-------------|-----------|
| Ti               | 24.8 (14.8) | 16.3 (9.2)  | 20.7 (7.0) |
| Coll             | 35.9 (14.1) | 12.4 (7.9)  | 25.3 (8.2) |
| Coll-nanofiber   | 47.1 (19.1) | 44.2 (16.2) | 45.7 (16.6) |

( ): SD
Connected bar: not significant ($p>0.05$)
No significant increase of the percentage of the gingival connective tissue contact was observed for the Coll-nanofiber implants at the mesial side due to the large deviation. The detailed reason is not clear. Observation at another direction such as buccolingual direction should be needed besides mesiodistal direction.

It remains unclear whether sprayed collagen nanofibers will degrade in vivo. If collagen nanofibers degrade, connective gingival tissue should face to titanium implant surface. Some technique for preventing the degradation of collagen nanofibers should be needed. A cross-linking treatment, which is used to control the degradation of collagen nanofibers, was not performed in the present study. Therefore, the influence of cross-linking on collagen nanofibers requires further investigation.

The ESD method can produce collagen nanofibers with different pore sizes or different diameters by varying some conditions, and these differences have an influence on the penetration of gingival connective tissue. Moreover, the ESD method may be applicable to other proteins such as laminin. The efficacy of laminin nanofiber- or collagen/laminin nanofiber-coated implants for gingival connective tissue attachment is of interest and warrants further study.

In conclusion, collagen nanofibers may have the ability to induce the generation of the gingival connective tissue attachment over the alveolar bone surrounding implants. Collagen nanofiber-coating is useful for improving gingival connective tissue response to titanium implants.

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