Original

Collagen Nanofiber on Titanium or Partially Stabilized Zirconia by Electro spray Deposition

Tohru Hayakawa¹⁾, Masao Yoshinari²⁾, Kazuya Nitta³⁾ and Kozo Inoue³⁾

¹⁾ Department of Dental Engineering, Tsurumi University School of Dental Medicine, 2-1-3 Tsurumi, Tsurumi-ku, Yokohama 230-8501, Japan.

²⁾ Division of Oral Implants Research, Oral Health Science Center, Tokyo Dental College, 1-2-2 Masago, Mihamaku, Chiba 261-8502, Japan.

³⁾ Fuenze Co., Ltd., 207 Wako RIKEN Incubation Plaza, 2-3-13 Minami, Wako-shi, Saitama 351-0104, Japan.

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Abstract: The present study demonstrated collagen nanofiber deposition onto titanium and partially stabilized zirconia, yttria-stabilized zirconia (Y-TZP), using an electro spray deposition (ESD) technique. Type I collagen was dissolved into 1,1,1,2,2,2-hexafluoro-2-propanol at different concentrations. The effects of the flow rate of the collagen solution and the applied voltage on collagen nanofiber deposition were investigated.

For titanium substrates, a collagen concentration of 50 mg/ml, a flow rate of 5.0 µl/min, and an applied voltage of 16 kV were found to be suitable for the deposition of uniform collagen nanofibers. Collagen nanofibers could be sprayed onto Y-TZP, which is not an electrically conductive substrate, although nanofibers fused during ESD. To avoid the fusion of nanofibers, titanium sputter-coating was performed on Y-TZP. Less fusion of collagen fibers occurred. A collagen concentration of 50 mg/ml, a flow rate of 5.0 µl/min, and an applied voltage of 25 kV were found to be appropriate for the deposition of uniform collagen nanofibers onto titanium sputter-coated Y-TZP. The ESD technique was shown to be effective for the deposition of collagen nanofibers.

Key Words: Collagen nanofiber, Electro spray deposition, Titanium, Partially stabilized zirconia

Introduction

Titanium and titanium alloys are widely used for dental and orthopedic implants, and various kinds of surface modifications, such as blasting or calcium phosphate coating, were reported to improve the bone response to titanium implants¹⁻³⁾.

It is predicted that the coating of titanium surfaces using cell-adhesive proteins enhances the cell activity or initial cell attachment around titanium implants, resulting in improved osteoconductivity. Recently, we developed a new technology for the immobilization of cell-adhesive proteins, namely, the tresyl chloride activation technique^{4,5)}. Previous study demonstrated that the immobilization of cell-adhesive proteins such as fibronectin or collagen onto titanium using the tresyl chloride method enhanced cell attachment, provided more spread morphologies of attached cells, and altered the gene expression profile^{6,7)}.

The electro spray deposition (ESD) method is a technique that involves spraying various kinds of solution containing biomacromolecules and/or synthetic polymers and causing them to form nano-sized particles and/or fibers. 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 shown in Figure 1. The ESD method consists of the following steps: A strong electric field is applied between a polymer solution contained in a glass capillary or syringe and a conductive substrate. When the voltage reaches a critical value, electrostatic forces overcome the surface tension of the solution, charged droplets are sprayed from the tip of the capillary into a dry atmosphere, and the dried droplets finally accumulate on the substrate to form a thin dry film of fine structures such as nano-/microscaled particles and fibers.

Morozov *et al.*⁸⁻¹¹⁾ deposited proteins by the ESD technique for protein microarrays and reported that the deposited proteins retained their biological properties. Protein films of α-lactalbumin were also deposited on poly(ethylene terephthalate), which was coated with aluminum, by the ESD technique. The response of deposited protein films to calcium ions was monitored as the biological activity of the films¹²⁾. Schouten *et al.*¹³⁾ deposited alkali phosphatase (ALP), calcium phosphate, or a combination of ALP and calcium phosphate coatings onto titanium and evaluated the in vivo bone response to different coatings. They concluded that ALP coatings formed by the ESD technique have potential as bone

Correspondence to: Dr. Tohru Hayakawa, Department of Dental Engineering, Tsurumi University School of Dental Medicine 2-1-3 Tsurumi, Tsurumi-ku, Yokohama, 230-8501, Japan Tel: +81-45-580-8365 Fax: +81-45-573-9599, E-mail: hayakawa-t@tsurumi-u.ac.jp

Fig. 1 Schematic drawing of electrospray deposition

Fig. 2 ESD apparatus used in the present study

implant coatings. Nanofibers of DNA, hyaluronic acid, citrosan, poly(lactic acid) or other synthetic polymers were also prepared by the ESD technique¹⁴⁻¹⁷.

As for a biomimetic nanofibrous extracellular matrix for tissue engineering, collagen and/or collagen/apatite and collagen/poly(glycolic acid) nanofibers were prepared by the ESD technique¹⁸⁻²⁴. Electrospun collagen nanofibers demonstrated cell growth, penetration of cells into the fiber matrix, and enhancement of cell attachment and proliferation^{18,19}. Cells grew not only on the top of the nanofibers but also into them. Collagen/apatite nanofibers increased the mineralization of osteoblast cells²¹, and collagen/poly(glycolic acid) fibers provided more elongation of adherent cells on fibers with smaller diameters²⁴.

However, there are few reports on the deposition of collagen nanofibers on titanium substrates. It is expected that the deposition of collagen nanofibers on titanium will promote better biological responses, such as the promotion of cell adhesion or bone formation. The aim of the present study was to deposit collagen nanofibers onto titanium substrates using the ESD technique. The effects of collagen concentration and applied voltage between capillary and substrate on nanofiber formation were investigated. In addition to titanium, partially stabilized zirconia, which has recently attracted attention as an esthetic material, was examined as a substrate. The effect of titanium sputter-coating on partially stabilized zirconia was also examined.

Materials and methods

Materials

As machined commercially pure titanium (Ti, JIS H4600, 99.9 mass%, Furuuchi Chemical Co., Tokyo) and yttria-stabilized zirconia (Y-TZP, Toso, Japan) disks with a diameter of 12 mm and a thickness of 1 mm were used. Type I collagen (Atelocollagen powder, Koken, Tokyo, Japan) was dissolved into 1,1,1,2,2,2-hexafluoro-2-propanol (HFIP, Wako, Osaka, Japan) at concentrations of 100, 50, 30, 15, and 7.5 mg/ml.

Titanium sputter-coating of Y-TZP was performed using an RF magnetron sputter method with a commercially available unit (High Vapor Deposition System, VEP-1000, ULVAC Inc., Kanagawa, Japan)²⁵. A titanium target (Furuuchi Chemical Co., Tokyo) with a diameter of 52 mm and a thickness of approximately 5 mm was used for sputter-coating. The target was bonded to a copper disc plate. The Y-TZP substrate was rotated in a stainless steel chamber (300 mm diameter x 500 mm height) at a rotational velocity of 30 rpm. The flow rate of argon gas was 30 sccm. The magnetron sputtering was performed using a radio frequency generator operating at 13.56 MHz at a power of 150 watts. The pressure of the chamber was maintained at approximately 0.3 MPa. After 10 min of sputtering, nitrogen gas was used to purge the chamber, which was then allowed to return to atmospheric pressure. Titanium sputter-coated Y-TZP (Ti-sputter/Y-TZP) was obtained. The film thickness, determined using a Dektak Stylus Profiler System (stylus force: 25 mg, scanning speed: 200 m/s, Veeco Instrument Inc., Woodbury, NY, USA), was approximately 0.02 μ m.

Electrospray deposition (ESD) of collagen nanofiber

Electrospray deposition (ESD) was carried out using an ESD device (Fig. 2, ES-2000, Fueno Co., Ltd., Japan). The collagen HFIP solution was sprayed from a glass syringe. The distance between the glass syringe and the substrate surface was 10 cm. The voltage applied between the glass syringe and the substrate was 16 or 25 kV, and the flow rate of the collagen HFIP solution was 0.5, 1, 2, or 5 μ l/min. The collagen HFIP solution was electrosprayed for 10 min. The nanofiber spraying was performed in an acrylic chamber under atmospheric conditions. The sprayed collagen nanofibers were then fixed in a vapor of 25% glutaraldehyde (Wako, Osaka, Japan) at 30 °C for 5 min. The conditions of the ESD experiments undertaken in the present study are listed in Table 1. The effects of substrate, concentration of collagen, flow rate, and applied voltage were investigated.

Fig. 3 SEM images of collagen nanofibers sprayed onto titanium using the ESD technique at a collagen concentration of 50 mg/ml (Experiment 1). The applied voltage was 16 kV.

a) Flow rate of 0.5 ml/min, b) flow rate of 1 ml/min, c) flow rate of 2 ml/min, and d) flow rate of 5 ml/min.

Table 1 ESD conditions for collagen nanofiber deposition

Code	Substrate	Concentration (mg/ml)	Flow rate (ml/min)	Voltage (kV)
Experiment 1	titanium	50	0.5, 1, 2, 5	16
Experiment 2	titanium	100	0.5, 1, 2, 5	16
Experiment 3	Y-TZP	50	5	16, 25
Experiment 4	Y-TZP	7.5, 15, 30	5	16
Experiment 5	Ti-sputter/Y-TZP	50	5	16, 25
Experiment 6	Ti-sputter/Y-TZP	30	5	16, 25

After fixation of the collagen nanofibers, the deposited nanofibers were coated with platinum and then the morphologies of nanofibers were observed using a field-emission scanning electron microscope (FE-SEM, JEOL, Tokyo, Japan) at an accelerating voltage of 5 kV.

Results

Figures 3 and 4 show the FE-SEM images of collagen nanofibers sprayed on titanium (Experiments 1 and 2 in Table 1). The effects of collagen concentration (50 mg/ml, Fig. 3 and 100 mg/ml, Fig. 4) and flow rate (0.5, 1, 2, and 5 μ l/min) were first examined. The applied voltage was 16 kV. When a collagen

concentration of 50 mg/ml was employed, the diameter of the collagen nanofibers was approximately 200-280 nm regardless of the differences in the flow rate. A greater flow rate tended to produce denser collagen nanofibers. At a flow rate of 5 μ l/min, the formation of dense and uniform collagen nanofibers was observed.

When the collagen concentration was increased to 100 mg/ml, the appearance of the fibers that formed was different from that at a collagen concentration of 50 mg/ml. The collagen nanofibers at a concentration of 100 mg/ml showed a less dense formation than those at 50 mg/ml. The nanofiber diameter increased to approximately 500-700 nm at a flow rate of 0.5 ml/min, and 200-

Fig. 4 SEM images of collagen nanofibers sprayed onto titanium using the ESD technique at a collagen concentration of 100 mg/ml (Experiment 2). The applied voltage was 16 kV.
a) Flow rate of 0.5 ml/min, b) flow rate of 1 ml/min, c) flow rate of 2 ml/min, and d) flow rate of 5 ml/min.

Fig. 5 SEM images of collagen nanofibers sprayed onto Y-TZP using the ESD technique at a collagen concentration of 50 mg/ml (Experiment 3). The applied voltage was 16 or 25 kV and the flow rate was 5.0 ml/min.
a) Applied voltage of 16 kV and b) applied voltage of 25 kV.

500 nm at a flow rate of 5.0 ml/min.

The surface appearances of collagen nanofibers deposited onto Y-TZP are shown in Figs. 5 and 6 (Experiments 3 and 4). The collagen concentration was 50 mg/ml and the flow rate was 5.0 ml/min. The applied voltage was 16 or 25 kV. Although Y-TZP is not an electrically conductive material, collagen nanofibers could be deposited on Y-TZP as shown in Fig. 5. However, the morphology of the collagen sprayed onto Y-TXP was different from that on titanium. Collagen fibers were fused and the fiber diameter was sometimes more than 1000 nm at 16 kV. When the

voltage was increased to 25 kV, the fusion of collagen fibers was not pronounced compared with that at a voltage of 16 kV. The fiber diameter was approximately 600 nm, which was still thicker than that on titanium, and collagen nanofibers deposited on Y-TXP were less straight and nanofiber spraying was less uniform than on titanium.

Then, collagen concentration was decreased in order to avoid the fusion of sprayed collagen fibers. The voltage was 16 kV. When the collagen concentration was 30 mg/ml, less fusion of collagen fibers was observed. At a concentration of 15 mg/ml,

Fig. 6 SEM images of collagen nanofibers sprayed onto Y-TZP using the ESD technique at different collagen concentrations (Experiment 4). The applied voltage was 16 kV and the flow rate was 5.0 ml/ml.
a) Collagen concentration of 30 mg/ml, b) collagen concentration of 15 mg/ml, and c) collagen concentration of 7.5 mg/ml.

Fig. 7 SEM images of collagen nanofibers sprayed onto titanium sputter-coated Y-TZP using the ESD technique at a collagen concentration of 50 mg/ml (Experiment 5). The applied voltage was 16 or 25 kV and the flow rate was 5.0 ml/ml.
a) Applied voltage of 16 kV and b) applied voltage of 25 kV.

Fig. 8 SEM images of collagen nanofibers sprayed onto titanium sputter-coated Y-TZP using the ESD technique at a collagen concentration of 30 mg/ml (Experiment 6). The applied voltage was 16 or 25 kV and the flow rate was 5.0 ml/ml.
a) Applied voltage of 16 kV and b) applied voltage of 25 kV.

nanofiber formation was not recognized, and a mixture of particles and thin nanofibers was observed at a concentration of 7.5 mg/ml.

Next, collagen nanofibers were sprayed onto Ti-sputter/Y-TZP (Experiment 5 and Experiment 6) as shown in Figs. 7 and 8. Ti-sputter/Y-TZP was electrically conductive. The collagen concentration was 50 mg/ml and the flow rate was 5.0 ml/min. The voltage was 16 or 25 kV. Nanofiber formation was more pronounced on Ti-sputter/Y-TZP (Fig. 7) than on Y-TZP (Fig. 5). However, some fusion of collagen fibers was still observed and the fiber diameter was also greater than that on titanium. Fiber diameter was approximately 400-500 nm. Comparing the results at different applied voltages, a higher voltage was found to produce less fusion of collagen nanofibers on Ti-sputter/Y-TZP than on Y-TZP. An applied voltage of 25 kV was found to be better for spraying collagen nanofibers onto Ti-sputter/Y-TZP.

When a collagen concentration of 30 mg/ml was employed, nanofiber formation was not clearly recognized. Fused nanofibers were observed together with deposited collagen films.

Discussion

In the present study, we deposited collagen nanofibers on titanium or on partially stabilized zirconia using the ESD technique. In the ESD process, the morphologies of the deposited nanofibers can be easily controlled by varying the applied voltage, solution properties such as concentration, viscosity, and surface tension, and the flow rate of the sprayed solution. The effects of collagen concentration, applied voltage, and flow rate of collagen solution on nanofiber formation were investigated. Moreover, the effect of titanium sputter-coating of partially stabilized zirconia substrate was examined.

First, the effects of collagen concentration and flow rate of collagen solution onto titanium substrate were examined. The present study revealed that a collagen concentration of 50 mg/ml produced more uniform and thinner nanofibers. Concentrations above 100 mg/ml could not be used for spraying collagen nanofibers during ESD because of the high viscosity of the collagen solution. Matthews et al.¹⁸⁾ reported that a concentration of 8 mg/ml of acid extracts of type I collagen (calfskin) did not result in any fiber formation using the ESD technique. Increasing the concentration of collagen to 83 mg/ml resulted in the formation of fibers during electrospinning. Further increases in collagen concentration did not grossly alter fiber formation. Rho et al.²⁰⁾ also used a collagen solution with a concentration of 80 mg/ml for the spraying of nanofibers. Our data suggest that a concentration of 50 mg/ml is suitable for the spraying of collagen nanofibers, which is close to the reported value of 80 mg/ml.

Low flow rates proved inadequate for the spraying of collagen nanofibers. A flow rate of 5 ml/min was found to be appropriate for the spraying of collagen nanofibers during ESD.

Partially stabilized zirconia has attracted attention as a material

for dental implants because of its esthetic properties and excellent mechanical characteristics²⁶⁻²⁹⁾. Yttria-stabilized zirconia, Y-TZP, was used as a substrate for collagen fiber deposition in the present study. Y-TZP is a material that is not electrically conductive. In principle, the ESD technique can only be applied for electrically conductive substrates. Electrically charged particles are typically sprayed onto an electrically conductive substrate. However, the present experiment demonstrated that collagen nanofibers could be sprayed onto Y-TZP material using the ESD technique. The thickness of the employed Y-TZP was 1.0 mm. It was speculated that a thickness of 1.0 mm did not prevent the deposition of electrically charged particles, although the critical thickness for materials that are not electrically conductive was not clear.

However, despite the above development, the fusion of nanofibers was observed. The charged collagen mist fused during spraying before its deposition on the Y-TZP substrate because Y-TZP is not an electrically conductive material. In order to avoid the fusion of collagen nanofibers, the applied voltage was increased to 25 kV and the collagen concentration was decreased. The increase in applied voltage and the decrease in collagen concentration to 30 mg/ml produced less fusion of nanofibers, but the formation of nanofibers was less uniform than that on titanium.

Titanium sputter-coating was applied to Y-TZP to improve its electrical conductivity. The effect of titanium sputter-coating was clear. More uniform collagen nanofibers could be sprayed at a concentration of 50 mg/ml and a voltage of 25 kV. Low concentrations did not result in a fiber structure being produced. The coating produced by titanium sputter-coating was very thin, approximately 0.02 mm, so the esthetic property of Y-TZP did not markedly deteriorate.

As a result, collagen nanofibers were determined to be suitable for deposition on not only titanium but also Y-TZP using the ESD technique. A collagen concentration of 50 mg/ml was found to be suitable for spraying collagen nanofibers. For titanium, an applied voltage of 16 kV is appropriate. On the other hand, an applied voltage of 25 kV is more appropriate for Y-TZP. Titanium-sputter coating was shown to be effective for the spraying of collagen nanofibers onto substrates that are not electrically conductive.

Further research is necessary to evaluate the biological effects of deposited collagen nanofibers by cell assays or animal implantation experiments. The ESD technique is a simple and convenient method for preparing protein nanofibers. Mixtures or laminates of different proteins can also be produced with this technique. It will be possible for other protein nanofibers, such as fibronectin or laminin nanofibers, and mixtures of collagen and fibronectin nanofibers to be sprayed on titanium and/or partially stabilized zirconia using the ESD technique.

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