A Bone-Like Precoating Strategy for Implants: Collagen Immobilization and Its Mineralization on Pure Titanium Implant Surface

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Many surface modification strategies are currently of interest in improving integration of implants to bone. An in vitro precoating of a bone-like mineralized layer of immobilized collagen on the implant surface is a potentially valuable approach to improve host acceptance of the implant. The goal of this investigation was to develop a method to precoat in vitro a bone-like mineralized collagen layer on a pure titanium dental implant surface. The study was conducted on acid-etched and nonetched surfaces of screw implants. Initially, a procedure was standardized to self-assemble collagen from a collagen solution. In subsequent experiments, the implant was also placed inside the solution, and after 3 days, collagen was found to be coated on the implant surface. Mineralization of the collagen gel as well as collagen coating on the implant was carried out by calcium phosphate precipitation from a mineralizing solution of calcium chloride containing polyvinyl phosphonic acid and polyaspartic acid, which served as polyanionic additives to help disperse the precipitation and template mineral nucleation. The implant was kept in the mineralizing solution and maintained for 2 weeks in an incubator at 37°C with a phosphate vapor phase generated from a vial containing dihydrogen ammonium phosphate in the incubator. Scanning electron microscopy, X-ray diffraction, and Fourier transform infrared spectroscopy analysis confirmed the coated layer to be a biomimetic bone-like mineralized type 1 collagen. Initial studies using osteoblast-like cells indicated cellular attachment on the modified surface. The method appears to be a promising way to generate in vitro a bone-like layer on the implant surface.

Key Words: collagen immobilization, titanium, implant surface, mineralization, bone-like coating

INTRODUCTION

Previous reports in the literature have shown that natural collagen scaffolds can be biomimetically mineralized.1–6 Typically collagen scaffolds are generally considered osteoconductive6 but not osteoinductive or osteogenic. Mineralization of such a substrate is believed to make the substrate also osteoinductive.8,9 Because mineralized collagen scaffolds contain both collagen and mineral constituents of bone in the local microenvironment of remodeling, they also have the potential to accelerate the biological remodeling in tissue regeneration. It has been shown in the past that collagen self-assembly occurs through a sol-gel transition from a collagen solution.10 This method can be potentially used to immobilize collagen on substrate surfaces. Mineralization of such deposited collagen on implant surfaces is a potential surface optimization method to provide a

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bone-like coating to improve conditions for osseointegration of an implant.

Commercially pure titanium and its alloys have been used successfully as endosseous dental implants for many years. It is also becoming an important material of choice for amputee prosthetics.11,12 The success of these implants is generally credited to the improved osseointegration involving the formation of bone in apposition to the implant placed within the bone. Despite the well-recognized successes of these implant systems in recent years, failure to achieve osseointegration is not uncommon in fixed prosthetic applications in dentistry, especially when the implant is placed in bone of problem areas such as low bone density (as is common in cancellous bone) or low vascularity (as may occur in cortical bone).

The potential for the success of osseointegration is largely determined by the biocompatibility and cellular response of implant surfaces critical for bone deposition. Pure titanium or its alloys, with or without a prepassivated or a hydroxyapatite-coated surface, are used in dental implant applications. Such surfaces are considered to be biocompatible, osteoconductive, and/or osteoinductive for facilitating cellular activity (osteoblast attachment, migration, and proliferation) for bone formation in apposition to implant surface. However, the continuing incidence of failures, especially in areas of poor bone density locations, indicates that these surfaces are not fully optimized, and additional research is warranted to improve the current state of the art in the surface structure of dental implants.

Biomimetic modification of titanium dental implant surfaces may potentially help improve their integration to bone. This promise has triggered a search for novel methods to modify the implant surfaces to improve osseointegration. Several approaches have been reported in the past. Such studies have been directed to explore the effect of surface modifications on osseointegration, and some reported studies are indicated below:

- changes in topography and microstructural features of the surface;13–18
- precoating hydroxyapatite on the implant surface;19
- organoapatite coating on the implant surface;20–23
- micro-arc oxidation24 and anodic oxidation25,26 of the implant surface;
- fluoride modification of implant surface;27
- RGD peptide and GRGDSP-peptide coating of implant surfaces;28–30
- hydrogen plasma cleaning; and21
- use of bone morphogenetic proteins (BMP-3).32–35

None of the previously reported methods used a surface modification to precoat a mineralized collagen structure (which is a bone-like structure) on the implant surface. Preforming such a bone-like coating on the implant in vitro before its placement in the bone may help accelerate osseointegration by facilitating a remodeling of the precoating to a natural bone structure after in vivo placement. Previous studies have shown that commercially available collagen sponge such as Cellagen is mineralized in a calcium chloride mineralizing solution using polyvinyl phosphonic acid (PVPA) and polyaspartic acid (PAA) as templating molecules for mineral nucleation.5,6 It was felt that this method of mineralization could be potentially combined with a sol-gel assembly of collagen fibers formed as a biomimetic collagen fiber coating on an implant surface to enhance osseointegration. The main objectives of this study were (1) to optimize a sol-gel method to prepare a collagen gel in a solution and apply the same method to deposit such a gel coating on an implant surface; (2) to mineralize the gel to form a biomimetic bone for tissue regenerative applications; and (3) to preform a collagen coating on a commercially pure titanium implant surface and to mineralize the coated layer to create bone-like precoating that may promote improved osseointegration.

**MATERIALS AND METHODS**

**Optimization of collagen self-assembly from a solution and its mineralization protocol**

The method of Pederson et al4 was used to form self-assembled collagen gel in a solution. Acid-soluble collagen from calfskin (MP Biomedicals, Aurora, Ohio) was used in the study. A collagen stock solution (1 mg/mL) was prepared by dissolving the collagen in fresh 0.1% acetic acid at 4°C for a minimum of 2 days. A 2:1 mixture of HEPES buffer containing 50 mM HEPES, 0.75 M NaCl, and 0.75M KCl with 0.28M NaOH was used to adjust the pH of the collagen solution to 7.4 to initiate gelation. The stock solution was prepared and stored at 4°C in a refrigerator, and then 100 mL of the collagen solution was placed in a dish. The pH was adjusted to 7.4 by adding HEPES buffer mixture, and the solution was also kept at 4°C in the refrigerator.

After 4 hours, a part of the gel formed by self-assembly was extracted and examined in a scanning electron microscope (SEM; Model S-2500, Hitachi, Tokyo, Japan) after critical point drying and sputter coating with gold. The major part of the gel was
subjected to mineralization. Mineralization was carried out by calcium phosphate precipitation from a preoptimized mineralizing solution using a 5-mmol calcium chloride solution containing 225 μg each of PAA and PVPA. The PAA (Sigma, St Louis, Mo, catalog No. P3418, M_w = 6200) and PVPA (PolySciences, Warrington, Pa, catalog No. 24297, M_w = 24 000) were used as polyanionic additives to help disperse the precipitation through charge-induced phase separation and to potentially act as mineral nucleation templates. The self-assembled collagen gel was kept in the mineralizing solution and maintained at 37°C in an incubator together with an adjacent vial in which diammonium hydrogen phosphate was kept as a source for establishing a phosphate vapor phase in the chamber. The precipitation process was monitored as a function of time. Optical microscopy (OM), SEM, X-ray diffraction (XRD), and Fourier transform infrared spectroscopy (FTIR) were used to study collagen self-assembly and mineralization features.

**Preliminary in vitro assessment of osteoblast attachment to collagen**

Samples of the mineralized collagen were also evaluated in a preliminary study to assess their ability to support cellular activity involving osteoblasts in cell culture using TE-85 osteosarcoma cells (American Type Culture Collection, Manassas, Va). Collagen gel before mineralization was used as a control. Specimens were seeded with suspensions of the TE-85 cells at 10 000 cells per cm² and maintained under standard cell culture conditions (5% CO₂, saturated humidity, 37°C) in a growth medium containing 10% fetal bovine serum. After 24 hours, the substrates were subjected to critical point drying procedures, and the samples were examined in an SEM (Hitachi S-2500). Experiments were carried out in triplicate to assure reproducibility.

**Collagen precoating on titanium implant surface and its mineralization**

The method of collagen self-assembly described previously was also used to form an immobilized layer of type 1 collagen fiber network on commercial titanium implant surfaces by keeping the implants immersed in the collagen solution during the experiments. The implants used were: (1) Steri-Oss implant (Yorba Linda, Calif) and (2) acid-etched Swede Vent dental implant (Core Vent Corp, Encino, Calif). The Steri-Oss implant is manufactured with a machined coronal portion (no acid etch) and a threaded implant portion, which is acid etched to generate a rough surface to promote osseointegration. Both of these surfaces were evaluated. A sample size of N = 3 was used to ensure reproducible results. Well-coated layer of collagen on the Swede Vent implant surface was also processed for in vitro mineralization by using the protocol optimized for mineralizing the collagen self-assembly described previously. The implant surface was examined visually and by SEM to characterize morphologic changes on the surface as well as by FTIR, energy dispersive spectroscopy, and X-ray diffraction studies.

**RESULTS**

Figure 1a shows an SEM micrograph of self-assembled collagen gel formed from the collagen solution, similar to the collagen fibrils seen in natural collagen. When samples of self-assembled collagen were mineralized using the calcium chloride–PAA-PVPA solution, the fibrils are coated with a dense layer of mineral phase, as seen in the SEM micrograph shown Figure 1b.

Figures 2a and b show the FTIR spectra of the self-assembled collagen gel and that of its mineralized structure, respectively. The strong absorption peak at 1000 cm⁻¹ in Figure 2b indicates the presence of phosphate in the mineral phase. The crystal structure of the calcium phosphate phase was revealed through X-ray diffraction in Figure 2b. The X-ray diffraction peaks confirm calcium phosphate deposition on the collagen scaffold, as indicated by the peaks closely representing hydroxyapatite superimposed on a diffuse spectrum due to the collagen matrix.

Figures 3a and b are micrographs of the collagen gel (before and after mineralization) after 24 hours of cell culture experiments with TE-85 osteosarcoma cells. Although the control specimen before mineralization (Figure 3a) gives no evidence of osteoblast proliferation or attachment, the micrograph in Figure 3b gives preliminary evidence that the mineralized collagen surface promotes cellular attachment, as evidenced by the tendency of the cells to adapt and attach to the sample surface through cell spreading and focal adhesion contacts (see arrows). Future additional experiments are needed for a detailed analysis of other markers of osteogenesis using quantitative alkaline phosphatase activity, osteoblast cell counting, expression of osteocalcin (a specific protein marker for mature osteoblasts), and so on to better understand the cellular activity on mineralized collagen.

Figures 4a and b are SEM micrographs of the surface of a coronal part of Steri-Oss commercially pure titanium sample surface before and after collagen self-assembly experiments. This surface is typically a machined surface with no acid etching as
the Steri-Oss implant is only acid etched on the threaded implant surfaces but not on the coronal portion. Although isolated collagen fibers were observed on the surface after collagen self-assembly experiments in Figure 4b, the surfaces were by and large devoid of uniform collagen deposition. In contrast, the acid-etched threaded surfaces were uniformly coated with collagen over the entire surface, as seen by comparing Figures 4c and d showing surface micrographs before and after collagen immobilization experiments. Similarly, the screw vent titanium implant surfaces before and after collagen deposition are shown in Figures 4e and f, and it is also uniformly coated with collagen fibers (Figure 4f). The results show that surfaces roughened by acid etching promote collagen immobilization, but the machined surface does not.

In addition, Figure 5 shows the micrograph after mineralization of the coated collagen layer on the screw vent implant surface. Figures 6a and b show XRD spectra of titanium implant obtained before (a) and after (b) biomimetic (mineralized collagen) coating on the Swede Vent implant surface. The additional peaks corresponding to hydroxapatite observed in Figure 6b, together with the diffuse spectral features, confirm hydroxyapatite deposition on an immobilized collagen layer on the titanium implant during mineralization experiments.

**DISCUSSION OF RESULTS**

Osseointegration of titanium implants results from the ability of titanium implant surfaces to promote osseointegration through cellular activity. In the past many approaches have been pursued to improve or accelerate osseointegration through various surface modification methods. In this study, we sought to develop a method to deposit in vitro a bone-like collagen-mineral composite layer on a titanium implant surface. Previous reports in the literature have shown that proper surface modification of the implant surfaces is a valuable approach to promote osseointegration. A biomimetic bone-like layer preformed on the implant surface should be considered as a valuable adjunct to the available surface modification methods.

In this study, we have developed a method to mineralize a collagen gel formed by self-assembly from a solution, and the approach was applied to coat a collagen-mineral composite layer on a titanium implant surface. It was expected that the implant surface condition should influence collagen deposition on the implant surface. We compared machined and acid-etched dental implant surfaces for the study. The coronal portion of the Steri-Oss implant surface, not pre-etched with acid to form a passive layer, was found to be unsatisfactory for uniform collagen deposition. On the other hand, the acid-etched threaded surfaces of Steri-Oss and Swede Vent implants promoted collagen immobilization on the respective surfaces. However, additional research is needed to characterize the relationship between different surface conditions of titanium implant and its ability to promote collagen self-assembly on the surface. The effect of rough surface morphology introduced mechanically, passivation through acid treatment, and/or anodic polarization methods on experimental groups should be assessed on collagen deposition in future studies.

The mineralization method we used was a biomimetic approach based on 2 known features of biomineralization in the body. One of these features is the sequestration of nucleation events in microscopic compartments known as vesicles and the other nucleation events templated by anionic protein molecules as templates. In our study, we used polyanionic molecules (eg, PAA and PVPA) to compartmentalize mineralization and template calcium phosphate nucleation. Compartmentalization was achieved through dispersion of acidic polymer molecules and is manifested by liquid-liquid phase separation with time due to the initial tendency for the negatively charged molecules to be dispersed in the solution, and the subsequent calcium enrichment and calcium phosphate formation in the dispersed regions. The molecules also potentially function as templates for mineral nuclei formation. It has been shown in previous reports that the liquid-liquid phase
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separation eventually causes nano-sized amorphous calcium phosphate particulate formation in the dispersed regions, and that these particles eventually become submicron-sized organo-apatite crystals with time extending to several days in the solution. The collagen substrate immobilized on the implant surface appears to be a receptive surface for such a mineralization process.

Our studies also indicate that collagen-mineral composite layer promotes cellular activity of osteoblast-like cells, and we are presently conducting more detailed investigations to be reported later. Although type 1 collagen is osteoconductive, it is typically deficient in osseoinductive expression, and in vitro mineralization promotes osteoblast cellular activity, as has been reported in the past. A precoating of mineralized collagen on implant surfaces provides the organic and inorganic constituents of bone structure in the local microenvironment of remodeling events in the host structure, and thus may potentially promote and accelerate osseointegration. The use of mineralized scaffold for bone-graft applications has a greater potential for long-term success if the mineralized structure has the same structure present in the tissue itself, as effective tissue remodeling process may depend on gross architectural match between the bone graft substitute material and the tissue. Thus, the strategy to immobilize a mineralized collagen layer on implant surfaces appears to be a promising approach to promote and accelerate osseointegration, but this needs to be further confirmed by careful additional in vitro and in vivo experiments.

**Conclusions**

A method to self-assemble and mineralize collagen gel and to precoat a bone-like layer of mineralized collagen immobilized on titanium implant surfaces has been demonstrated. The mineralized layer was found to promote cellular activity, indicating potential for more efficient bone remodeling at the implant-tissue interface. This may promote and/or accelerate osseointegration.

**Figures 4 to 6.**

Figure 4. Scanning electron microscopy micrographs of collagen layer and its mineralization. (a) Initial coronal surface of Steri-Oss implant as is, (b) coronal surface of Steri-Oss implant after collagen coating, (c) acid-etched (threaded region) surface of Steri-Oss implant before collagen coating, (d) acid-etched (threaded region) of Steri-Oss implant surface after collagen coating, (e) initial surface of Swede Vent implant, and (f) Swede Vent implant after deposition of immobilized collagen layer on the implant surface, before mineralization.

Figure 5. Swede Vent implant surface after mineralization experiments. Figure 6. X-ray diffraction patterns of Swede Vent implant before (a) and after (b) mineralized collagen coating. Note the additional hydroxyapatite peaks in (b) confirming mineral deposition.
ACKNOWLEDGMENT

Partial support of this study by NIH-NIDCR grant No. R01DE14370 is gratefully acknowledged.

REFERENCES

33. Hartwig CH, Esenden WA, Pfund A, Kuuswetter D, Herr G. Improved osseointegration of titanium implants of different surface characteristics by the use of bone morphogenetic protein
