Fabrication of bioactive glass coating on pure titanium by sol-dip method: Dental applications

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This study aimed to assess the mechanical and biological properties of bioactive glass (BG) coating on titanium (Ti). Bioinert Ti substrates were coated by BG to induce bioactivity to the surface. The sol-gel derived BG 58S sol was successfully prepared and coated on the abraded and blasted Ti surface using the sol-dip method. The characterization and cell study for all substrates' surface was carried out. Adhesion test confirmed that a firmly adhered BG coating layer was formed on the abraded and blasted Ti. The measured bonding strength between the coating and the blasted Ti substrate was the highest among all samples, which was 41.03±2.31 MPa. In-vitro cell viability and alkaline phosphatase activity (ALP) tests results also showed that BG coating on the Ti substrate improved the biological properties of the surface. The BG sol-dip coating method could be used to fabricate Ti substrate with a bioactive surface.

Keywords: Ti, Bioactive glass, Coating, Sol-dip, Adhesive strength

INTRODUCTION

Ti has been applied as a metallic biomaterial in dentistry by Brånemark et al.1. Ti and its alloys have substantial superiorities in comparison to other metals such as stainless steels and Co-Cr alloys because of their excellent biocompatibility, non-sensitization of tissues, good mechanical properties and low density2,3. However, the Ti substrate is known as a relatively bioinert surface which tends to form a fibrous tissue layer at the interface between the implant and bone4-6.

Recently, the coated Ti has been applied in implant surgeries because the underlying metal has excellent mechanical and biological properties, while the calcium phosphate coating enhances the bonding of implants to the enclosing bone7,8. Further, chemical modification of the Ti implant surface with bioactive materials has led to important achievements9-10. BG is known to have several unique properties, such as bioactivity, biodegradability, bone regeneration ability and direct bone contact ability11. The bioactivity of the BG coating could be due to calcium and phosphorous release12,13. On the other hand, BG can firmly bond with the host tissue by forming a bone-like apatite layer14. BG was introduced, for the first time, by Larry Hench at the University of Florida in 196915; the sol-gel derived BG 58S was reported by Li et al. in 199116. Ceramic BG 58S containing silica 58%, calcium oxide 33% and phosphate 9% metal oxides could be fabricated by different techniques; the sol-gel preparation procedure was used in this study.

Several methods have been applied for the coating of BG on the Ti substrate, such as pulsed laser deposition17, hydrothermal method18, dip-coating19, plasma spray20; each method has its advantages and disadvantages.

Sol-dip coating method is a well-known technique for BG coating due to the ease of glass preparation, quick coating procedure and the homogeneous coating layer21,22. Coefficient of thermal expansion is a parameter preventing the coating of metals at high temperatures23. However, sol-dip coating is processed at low temperatures. Also, the sol-dip coating method is able to cover the complex Ti surface with a uniform thickness of coating materials24. Besides, the coated materials on the implant metal surface should not be collapsed after implantation into body, and surface pre-treatment with alumina blast before the coating procedure creates a perfect condition enhancing the bond strength between the coating layer and substrate25,26.

The objective of the present study was to fabricate and assess a bioactive and firmly adhered BG 58S coating on the Ti substrate by utilizing the sol-dip coating method, as well as investigating the effect of the Ti surface roughening on the coating properties.

MATERIALS AND METHODS

Preparation of the Ti samples

Commercially available medical grade Ti plates with the...
The air abrasion (alumina blasting) of the Ti samples was carried out using a blasting machine (Jet Blast III, J. Morita Mfg., Osaka, Japan). The alumina abrasive powder (Hi-alumina, Shofu, Kyoto, Japan) with a mean particle size of 50 μm was used under the pressure of 0.5 MPa. The Ti substrates and the head of the nozzle distance and time were set to 20 mm and 30 s, respectively; the samples were moved gently to obtain a homogenous rough surface. The abraded and blasted samples were washed and cleaned in acetone, ethanol and distilled water with an ultrasonic cleaner for 15 min per each and then dried in an oven at 37°C for 1 h.

Preparation of the BG 58S sol
The chemicals, ethyl tetraethyl orthosilicate (TEOS) (Fujifilm, Wako Pure Chemical, Osaka, Japan), triethyl phosphate (TEP) (Wako), calcium nitrate tetrahydrate (Ca (NO₃)₂.4H₂O) (Wako), and deionized water (DW), were used to prepare the BG 58S Sol. The preparation method of BG 58S was according to the study by Lei et al. Accordingly, 10.24 gr TEOS, 1.16 gr TEP and 7.02 gr (Ca (NO₃)₂.4H₂O) were added into the mixture of 7.82 mL DW and 1.30 mL HCL (2M); then, the mixed solution was stirred for 3 h to become a uniform sol.

BG sol-dip coating
The prepared Ti samples were gently coated with the BG solution by the sol dip-coating method under the following condition: dip length: 60 mm, dip speed: 9 mm/s, Return speed: 9 mm/s, dip duration: 4 s and dry duration: 60 s. After coating the samples, the BG-covered substrates were aged for 72 h in the incubator at 37°C to make the hydrolysis of polycondensation. Then, the coated samples were transferred to the oven, which was set at 60°C and 120°C for 24 h per each, respectively, to remove the excess water and ethanol from the surface. Finally, the samples were put at a 650°C furnace for 1 h to remove the unreacted organic matter.

Preparation of the cross-section samples
The abraded Ti, BG-coated abraded Ti, blasted Ti, and BG coated blasted Ti samples were embedded in the epoxy system (SpeciFix-20; Struers, Ballerup, Denmark). The mounted specimens were cut and polished with 400# grit abrasive paper for preparation of the cross-section samples.

Morphology, phase and elemental analyses
Surface and cross-sectional morphologies of the abraded and alumina blasted Ti before and after BG coating were observed by field emission scanning electron microscope (FE-SEM, JSM-7500FAM, JEOL, Tokyo, Japan); also, the surface and cross-section elemental analysis of the BG coated Ti before and after blasting were evaluated using an energy-dispersive X-ray spectroscope (EDS, JSM-7500, JEOL). Phase structure of the BG 58S and abraded and blasted Ti before and after BG coating was analyzed by X-ray diffractometer (XRD) (Miniflex600, Rigaku, Tokyo, Japan). The diffractometer operated at 40 kV and 15 mA, with a 20 range of 20°–80°, by utilizing a step size of 0.02.

Mechanical properties
The adhesion strength of the abraded and alumina blasted Ti samples before and after BG coating was evaluated using a universal testing machine (Instron 5566S, Instron, Canton, MA, USA) at a crosshead speed of 2 mm/min. Aluminum stud-pins with the diameter of 2.7 mm, which were covered by a special reinforced epoxy resin adhesive (ROMULUS IV, Epoxy Resin Adhesive, Quad Group, Spokane, WA, USA) with the thickness of 50 μm, were clipped on the surface of the samples and aged in the oven at 150°C for 1 h. The adhesion strength of five samples in four different groups, named as abraded Ti, BG-coated abraded Ti, blasted Ti and BG-coated blasted Ti, was tested. The measured and calculated average values and the standard deviation of the tested samples were evaluated.

Cell viability
In this study, a preosteoblastic cell line MC3T3-E1 provided by Riken, a Japanese comprehensive research institution, was used for the in-vitro evaluation of the uncoated and coated samples. Cells were cultured in an alpha-minimum essential medium (α-MEM, GIBCO, Invitrogen TM, NY, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO), 100 units/mL penicillin and 100 µg/mL streptomycin (Wako) in a humidified 5% CO₂ balanced-air incubator at 37°C. Cell viability of the uncoated and BG coated Ti samples was measured by the MTS assay (Cell Titer 96AQueous Promega, Promega, Madison, WI, USA). All samples were placed in the 24-well cell culture plates and the MC3T3-E1 cells were seeded onto the samples with a density of 1×10⁴ cells/well. The plates were incubated for 3, 6 and 9 days and the proliferation medium was changed every two days. After each incubation period, 50 µL of the MTS solution was added to each well containing medium; then, the plates were incubated for 4 h at 37°C in the 5% CO₂. The medium was transferred to the 96-well plate and the absorbance was measured at the 492 nm wavelength by the plate reader (Multiskan™ FC, Thermo Fisher Scientific, Waltham, MA, USA). Five specimens were tested for each group and incubation time. The data were evaluated using one-way analysis of variance (ANOVA) with the statistical significance set at p<0.05.

Alkaline phosphatase activity (ALP)
Differentiation of seeded cells on the substrate materials was evaluated by measuring the alkaline phosphatase (ALP) activity. The differentiation medium was prepared by the addition of 50 mL FBS, 50 µg/mL ascorbic acid, 10 mM β-glycerophosphate and 50 nM dexamethasone to the growth medium. The samples, before and after BG coating, were placed on 24-well plates and 1×10⁴
cells were seeded on them. The plates were incubated for 7, 14 and 21 days and the differentiation medium was changed every two days. The ALP kit (Wako) was used to evaluate the differentiation of MC3T3-E1 cells in direct connect with the substrate materials on each cultured day. The cells were lysed by extraction solution for 15 min; then, the substrate solution was added and incubated at 37°C in the 5% CO₂ for 45 min. This was followed by adding the stop solution to prevent the reaction. The medium was transferred to a 96-well plate and measured by the plate reader (Multiskan™ FC, Thermo Fisher Scientific) in the 405 nanometer wavelength. Five specimens were tested for each group. The data were evaluated using one-way analysis of variance (ANOVA), with the statistical significance set at p<0.05.

In-vitro mineralization
The calcium nodule formation ability of the samples was investigated using alizarin red S (Wako). The MC3T3-E1 cells were seeded on the uncoated and coated substrates in a 12-well plate at the density of 1x10⁴ cells/well. The plates were incubated at 37°C in the 5% CO₂ for 7, 14, 21 and 30 days. After each incubation period, the cells were stained in the alizarin red solution. The samples were washed with PBS and then stained with 5% alizarin red S for 15 min. The samples were washed with deionized water to remove the excess staining reagent. The stained samples were observed and photographed using the digital microscopy (VHY-100K, Keyence, Tokyo, Japan).

RESULTS

Surface morphology and elemental analysis
Figure 1 shows the FE-SEM images, elemental analyses and chemical composition of the abraded Ti, BG-coated abraded Ti, blasted Ti and BG-coated blasted Ti. The FE-SEM images confirmed that the BG coatings were formed on the abraded (Fig. 1b) and blasted (Fig. 1d) Ti surfaces. The topographical information provided by FE-SEM showed a uniform BG coating layer on the rough blasted Ti surface. EDS results also demonstrated the existence of Ti, Ca, P, and Si elements on the surface after BG coating on the abraded (Fig. 1b) and blasted (Fig. 1d) Ti surfaces. Also, the aluminum (Al) element could be detected after blasting on the Ti surface, because of alumina particles collision to the surface. However, the intensity of the detected Al element was lower than that of the uncoated Ti after BG coating.

cross-section examination
Figure 2 exhibits a cross-sectional microstructure and elemental analysis of the abraded Ti, BG-coated abraded Ti, blasted Ti and BG-coated blasted Ti. The FE-SEM images for the BG-coated abraded and blasted Ti (Fig. 2b and Fig. 2d) showed the BG coating full coverage of the Ti substrate and the interface between metal and ceramic obviously. Also, the EDS spectrums for the BG-coated abraded and blasted Ti confirmed that the existence of the Ca, P and Si elements in the interface between the Ti substrate and BG coating. Also, the Al element was detected for the blasted Ti sample before and after BG coating (Fig. 2c and Fig. 2d).

Phase structure
Figure 3 exhibits the XRD patterns of the BG powder, the abraded Ti, the BG-coated abraded Ti, the blasted Ti and the BG-coated blasted Ti. The BG powder showed a pattern similar to the previous studies with an amorphous phase and amorphous peak. The Ti peaks were observed in all un-coated and coated substrates. The Ti peak intensity was decreased in the BG coated samples. In the XRD pattern of the BG coating layer on the blasted Ti, the apatite phase was assigned. The XRD patterns of the BG coated surfaces (Fig. 3c and Fig. 3e) confirmed that the apatite peaks were distributed at 20 of 26° and 32°. The apatite peak intensity was higher in the BG-coated blasted Ti substrate.
Fig. 2 FE-SEM and EDS results of the cross-section samples, abraded Ti (a), BG-coated abraded Ti (b), blasted Ti (c) and BG-coated blasted Ti (d)

Fig. 3 XRD patterns of BG 58S powder (a), abraded Ti (b), BG-coated abraded Ti (c), blasted Ti (d), and BG-coated blasted Ti (e)

Fig. 4 The adhesive strength of the abraded Ti, BG-coated abraded Ti, blasted Ti and BG-coated blasted Ti substrates (*p<0.05)

Fig. 5 The proliferation of MC3T3-E1 cultured on abraded Ti, BG-coated abraded Ti, blasted Ti and BG-coated blasted Ti (*p<0.05)

Mechanical properties
Figure 4 demonstrates the adhesion test results of the abraded Ti, BG-coated abraded Ti, blasted Ti and BG-coated blasted Ti substrates. The measured adhesive strength for the abraded Ti, BG-coated abraded Ti, blasted Ti and BG-coated blasted Ti substrates was 45.88±1.20, 35.04±0.55, 50.06±0.96, 41.03±2.31 MPa, respectively. The adhesion test results confirmed that the BG coated samples’ adhesive strength had been reduced. Moreover, the BG-coated blasted Ti substrate adhesive strength showed a significant difference in comparison to the BG-coated abraded Ti substrate.

MTS assay
Figure 5 expresses the results of the MTS assay. Cell viability proved that the cells had been proliferated for all samples after 3, 6 and 9 days, except the abraded Ti, after 6 days. The results also showed that there was a significant difference in terms of the proliferation of MC3T3-E1 cultured on both abraded Ti and blasted Ti, and BG coating groups after 9 days.

ALP activity
Figure 6 shows the ALP activity of MC3T3-E1 cultured in direct contact on each group of the substrates: abraded Ti, BG-coated abraded Ti, blasted Ti and BG-coated blasted Ti. According to the statistical analysis of the ALP activity results, the BG coated samples in both abraded and blasted Ti showed a significant difference with the uncoated samples (abraded and blasted Ti)
**Fig. 6** The differentiation of MC3T3-E1 cultured on the abraded Ti, BG-coated abraded Ti, blasted Ti and BG-coated blasted Ti (*p* < 0.05)

**Fig. 7** Calcium nodules formation of MC3T3-E1 cells cultured on the abraded Ti and Ti blast samples before and after BG coating upon 7, 14, 21 and 30 days of the incubation time.

after 14 and 21 days of differentiation.

**Calcification**

Figure 7 shows the biomineralization of the MC3T3-E1 cells on the abraded Ti, BG-coated abraded Ti, blasted Ti and BG-coated blasted Ti samples after 7, 14, 21 and 30 days of differentiation. The results of alizarin red staining for calcium deposition on the MC3T3-E1 extracellular matrix in all groups and at each incubation time showed that the calcium nodules formation for the BG-coated abraded Ti and BG-coated blasted Ti after BG coating was more than that in both abraded and blasted Ti.

**DISCUSSION**

**Morphology, phase and elemental analysis**

Studies have shown that mechanical and biological properties are not inherent features of materials; this means that these properties are the functions of the materials and they could be improved by utilizing different techniques. Besides, the biological properties of materials can be improved by applying various chemical modifications on the surface of them. Thus, in the present study, the chemical modification of the Ti substrate was applied by the formation of a bioactive coating layer (BG) on the surface. The surface morphology results of the samples showed that a typical BG coating layer could be made on the Ti substrate by using the sol dip-coating method (Figs. 1b and d). The FE-SEM image exhibited that the BG coating was homogeneously covered the Ti surface. The uniform coating of the BG layer could be seen on the Ti substrate after sol dip-coating on the blasted Ti substrate. Blasting has a great ability to produce many micro and nano-scale cavities on the Ti surface, which are directly related to the induced bond strength. The FE-SEM image obtained for the blasted Ti substrate showed that the rough blasted Ti surface was fully covered after BG coating. The EDS results (Fig. 1) demonstrated that Ca, P and Si elements were detected on the surface after BG coating on the abraded and blasted Ti substrates. The Ca and P elements on the BG-coated blasted Ti samples were more than those in the BG-coated abraded Ti surface; this was due to the thicker BG coating layer on the BG-coated blasted Ti sample. Moreover, the Al element was detected on the blasted Ti substrate due to the usage of the abrasive alumina powder in this method. However, after BG coating on the blasted Ti, the amount of the detected Al element was reduced dramatically. From the cross-sectional FE-SEM images it was clear that the Ti surface was fully covered with BG. On the other hand EDS results of the BG-coated abraded Ti and BG-coated blasted Ti samples (Figs. 2b and d), it has been determined that Ca, P and Si elements were detected in the interface between Ti and BG coating layer. The XRD peaks (Fig. 3) were assigned to the two main phases of Ti and apatite. The apatite was detected because BG had the ability transform to this phase. Also, small peaks for alumina and the crystallized apatite were detected. No incognito peaks were, however, detected in this experiment.

**Mechanical properties**

An ideal coating for dental implant applications should have excellent mechanical properties and optimum adhesive strength without collapsing after implantation into the body. In the present study, we conducted an adhesion test in order to evaluate the bonding strength of the coating layer to the abraded Ti and blasted Ti substrates before and after BG coating, obtaining noteworthy results (Fig. 4). In the BG preparation procedure, since it had to be heated at 650°C, the Ti oxide phase in the Ti and BG interface could be transformed from anatase to rutile; therefore, this reaction could contribute to the rise of the BG and Ti adhesion. Dental application of materials required a high mechanical properties due to the loading condition in the mouth. Therefore, in the coating layers it is necessary to have high adhesive strength to the bulk. Modified surface is
one of methods to improve the mechanical properties of the coating layers. Surface roughness is an important factor in the establishing of a reliable connection between the bulk and coating layer. Previous studies on titanium implants has shown that blasting is an effective method in the producing of a rough surface in order to increase the adhesive strength between coating layer and implants. Alumina blasting has the potential to produce many micro and nano-scale cavities on the Ti substrate that are directly related to the resulting bond strength. The air abrasion with alumina was utilized to create a rough surface and prevent the reduction of mechanical properties. The key role of the alumina blast in maintaining and preventing the reduction of the mechanical properties of Ti is well visible in the adhesion test results. According to the objective of this study, it was assumed that the blasting of the Ti implant substrate could prevent the reduction of the coating mechanical properties. Based on the literature, the rough surface can improve the biocompatibility and mechanical properties of the dental implants. Adhesion test results obtained in this study confirmed that there was a significant difference between the abraded and blasted samples results. The lowest adhesive strength was recorded in the abraded Ti samples after BG coating, while the highest one was recorded for the blasted samples. The measured adhesive strength for the BG layer coated on the blasted Ti was 41.03±2.31 MPa (p<0.05), showing that the BG coating was adhered firmly to the blasted Ti surface. On the other hand, the bonding strength of the BG-coated blasted Ti plates in our study was higher than that of the H12 glass coating on the Ti6Al4V plates for 36±2 MPa.

**Cell response**

The most important function of the dental materials used in implantation industry is stimulating the proliferation and differentiation of the dental cells; the dental material implants must be biocompatible and nontoxic with excellent mechanical properties. BG coating could improve the viability of the dental implant substrate; also, the BG coating biocompatibility is dramatically increased after the induction of the hydroxyapatite formation through coating. The MC3T3-E1 pre-osteoblasts cells were used for the in-vitro cell response assessment of the cultured cells on the abraded Ti, BG-coated abraded Ti, blasted Ti and BG-coated blasted Ti substrates. This was a biological in vitro study to evaluate proliferation, differentiation and mineralization of all uncoated and coated groups. The effect of both factors surface roughness and chemical treatment like BG coating on the proliferation has been confirmed. Also, in the present study, the MTS assay results after 6 and 9 days of the cell culture indicated that the viability of the abraded Ti substrates after BG coating was higher than that of the abraded Ti substrate before BG coating. The cell viability results after 6 and 9 days for the blasted Ti samples with BG coating was higher than that of the blasted Ti samples without BG coating. The most considerable point was comparison of the BG-coated abraded Ti and BG-coated blasted Ti groups; the viability for the BG-coated blasted Ti substrates was higher than that for the BG-coated abraded Ti. The viability results suggested that the cells had good proliferation in the chemically modified surface with a rough surface.

**ALP activity**

ALP activity is a well known specified phenotypic markers for osteoblastic cells which is indicating the mineralizing ability of cells. Alumina blasted Ti samples, after BG coating, showed the less inhibition of the ALP activity of MC3T3-E1 in comparison with its blasted Ti and BG-coated abraded Ti counterparts; therefore, this revealed the less inhibition of the ALP activity of MC3T3-E1, as compared with the abraded Ti. This also means BG coating materials, because of their compounds such as Ca and P, as confirmed by the EDS spectra, could transform the surface from a bioinert one to a bioactive one, thereby improving the biocompatibility and biological properties of Ti. Comparison of the results in two groups, BG-coated abraded Ti and BG coated blasted Ti, reported that MC3T3-E1 cells differentiation on the blasted surface was higher than that of the smooth abraded surface. Hydroxyapatite is the most important tooth enamel and dentin mineral; the human bone is a modified form of hydroxyapatite mineral. Besides, ALP activity is closely related to the mineralizing capacity of the cells, and apatite coating provides the substrate for further cells activity due to its characteristics. According to the XRD patterns of the samples, the apatite coating formation on the substrates after BG coating could be one of the parameters influential on the ALP results.

**Alizarin red S staining**

Alizarin red S staining has been applied to assess calcium-rich deposits by cells in the cell study experiments. The effect of Alizarin red S staining assay on the MC3T3-E1 cells in each incubation time confirmed that the BG materials elevated the calcium nodules formation of the cells. The cell culture procreated more calcium deposits after placing BG materials in their vicinity through coating on the substrates. This good event could be interpreted by the increase in the Ca content on the surface, which could be regarded as one of the major factors related to mineralization.

The results also suggested that the BG materials coating on the Ti implants surface had enhanced biological properties more than pure Ti by improving cell proliferation, raising the ALP activity and elevating the formation of the mineralized bone nodules. They also showed that Ca, Si and P containing components of the coated BG could promote the biocompatibility of Ti as a dental implant in the present study. Besides, our study showed the impressive effect of the rough surface on optimizing the typical coating on the substrate, enhancing the mechanical properties dramatically and to improving the biological properties. Further research is, however, recommended to study the mechanism of the effect of the BG materials on the dental implants surface.
CONCLUSION

Ti samples were successfully coated with BG 58S bioceramic by the sol dip-coating method. The firmly adhered coating could be made by the BG material on the pre-modified Ti implant. Surface modification of the implant with alumina blast and the subsequent BG coating improved the mechanical properties and improved the biological stability. Surface roughening is used to prevent problems such as the reduction of the mechanical properties of Ti implants after chemical treatment; so, it can contribute to the improvement of the biological properties.

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