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Novel Bioactive Glass as a Potential Clinical Appliance

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Abstract: This paper examined the biocompatibility with the bone of novel types of bioactive glass (AP40, RKKP) *in vivo*. And we investigated the possibility to clinical applications. For the experimental studies, mirror polished titanium, bioactive glasses (RRKP and AP40) and coated HA (hydroxyapatite) were prepared. The investigation period was 72 weeks and observations were conducted at the interface by toluidine staining (undecalcified section) and by TEM (transmission electron microscopy). As a result, novel bioactive glasses are considered as biomaterials that can be fully applied in clinical practice as bone filling materials, scaffolds for regenerative therapy, and coating materials for titanium and/or zirconia similar to Hydroxyapatite and Titanium.

Key words: Novel bioactive glasses, biocompatibility, *in vivo*, bone, possibility, clinical appliance.

1. Introduction

Various biomaterials were developed and studied in the 1970s, leading to the biomaterials that are currently in use. In the dental field, titanium alloy has been utilized clinically in dental implants. HA (hydroxyapatite) is used to coat titanium and fill bone defects. While bioglass materials have been applied clinically in the past, at present, they are not actively used due to their fragility. However, studies of bioglasses have continued, including research on Hench's glass, A/W glass, AP40, RKKP, RBP1 and RBP2.

For biomaterials to be used in the human body, not only the development of novel biomaterials but also a thorough evaluation of the biological response to the materials due to determine the biocompatibility is necessary.

In 1991, we developed a high-velocity frame coating technique for HA onto a titanium alloy in cooperation with Asahi Optical Co., Ltd. This technique involves, due to improvement of the crystallinity of coated HA, a 4-fold increase in coating speed by decreasing the spraying temperature compared with conventional plasma spraying methods in order to increase crystallinity. For its clinical application, we evaluated the biocompatibility *in vivo* in a one-year study and showed that our coated HA was extremely biocompatible with bone. Based on the results of a one-year long *in vivo* experiment, we successfully commercialized it and proceeded with the actual clinical application. Thus, we clinicians believe that it is important to consider the biocompatibility of the applied biomaterials over a long term.

In 1969, Hench first discovered a type of bioactive glass (Na_2O-CaO-P_2O_5-SiO_2) that he called 45S5. He reported the results of *in vitro* biocompatibility studies and *in vitro* tests of the interfacial bonding of implants to bone in 1971 [1-2]. He found that the mechanism for the development of this bond involves production of an amorphous ion surface gel on the bioglass. This gel induces osteogenesis through the chemotactic activity of osteoblasts. The glass then bonded to a layer of collagen fibrils produced at the interface by osteoblasts. The chemical bonding of the HA layer to collagen created a strongly bonded interface [3]. However, fragility was a problem. In 1990, Yamamuro and Kokubo [4] developed apatite-wollastonite (A/W) bioactive glass. This

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material contained 38% oxyapatite and fluoroapatite, 34% β-wollastonite ($CaO\text{-}SiO_2$) and 28% residual glass. However, manufacturing was difficult due to the hardness of the material. Recently, there have been various studies on AP40 and RKKP [5-11]. AP40 and RKKP exchange ions far more slowly than Hench's glass (e.g. 45S5). The difference between AP40 and RKKP is the presence of La (lanthanum) and Ta (tantalum). Small amounts of La_2O_3 and Ta_2O_5 (RKKP) were added as possible nuclei for the deposition of ions involved in bone formation. The slower exchange of RKKP promotes the formation of silicate chain networks as well as stabilizes and—more importantly—increases the packing density of the molecular network. *In vitro* experiments have shown that the presence of these oxides can modify the surface properties of the glass and influence protein absorption kinetics. In 2008, we used SEM (scanning electron microscopy) and TEM (transmission electron microscopy) to demonstrate for the first time *in vitro* that RKKP is the most biocompatible bioglass with human gingival epithelial cells (HGE-15 cells) [11].

Ravaglioli and Krajewski [10] developed RBP1 and RBP2 based on AP40. They reported that RBP1 and RBP2 were less stable than RKKP. Consequently, their liquidus temperatures, which indicate in a way the strength of the molecular bonds in the molecular network of the glass, are lower than that of RKKP. In addition, studies of the ionic release rates of RBP1 and RBP2 have shown that these glasses exchange ions with physiological solutions more slowly than AP40 or RKKP. A slower release produces smaller changes in terms of the ionic presence and the physicochemical variations around the implanted piece of bioactive glass, as well as a more highly charged positive/negative double layer. ZnO (zinc oxide) was added to both RBP1 and RBP2. ZnO is known as a cicatrizant agent, and Zn ions are useful for controlling the solubility of the glass system since they reinforce its structure. Sr (strontium) is related to hardness. Nb (niobium) has resistance to many chemical materials and is manufactured easily at low temperature. In RBP1 and RBP2, Zn^{2+}—which is much more active than Ta^{5+}/La^{3+} in RKKP—acts as a moderator of the ionic leaching rate [5,10].

Because artificial dental roots used in the dental field penetrate the gingiva and are embedded in the bone, we need to examine the biocompatibility with epithelial tissues and bone tissues, which have different origins (epithelial tissue is an ectodermal system, whereas bone tissue is a mesodermal system).

In 2014, we first examined the biological responses of the interface between new bioglasses (RKKP, RBP1, RBP2) and HGE (human gingival epithelial) cells using TEM *in vitro*. Only RKKP was found to directly bond to the cells without an intervening layer. In contrast, for RBP1 and RBP2, we observed a gel like layer at the interface on TEM photographs [12]. In 2017, we investigated the differences in the interface between new types of bioactive glass (RKKP, RBP1, RBP2), mirror-polished titanium alloys and a Plastic culture dish (as a control) for the evaluation of HOCs (human osteoblast cells) using TEM. In a Plastic culture dish, we observed focal contact, and close contact in the places between the HOCs and the Plastic culture dish. In M-Ti, we observed a non-structured homogeneous layer. In RBP1 and RBP2, an intervening layer with a gel-like consistency of approximately 100 nm in thickness was observed. In RKKP, the collagen fibers bonded directly, indicating the presence of a bone matrix of HOCs at all sites. Given these results, we concluded that RKKP was the best biomaterial, as it bonded directly to HOCs without an intervening layer.

The purpose of this study, based on the *in vitro* results using TEM thus far, is to examine the application of novel bioactive glasses in clinical practice by conducting *in vivo* implant experiments.

2. Material and Methods

For the experimental studies, mirror polished

titanium (1.0 mm diameter, 5.0 mm length; Kobe Steel Co., Ltd. Kobe Japan), bioactive glasses (fragments of RRKP and AP40; Institute of Science and Technology on Ceramic Materials, Faenza, Ravenna, Italy) and coated HA (1.0 mm diameter, 5.0 mm length, 50 μm coating thickness; Plasma Biotal Co., Ltd., U.K) were prepared.

White adult male rabbits of approximately 3.0 kg in body weight were anaesthetized by intravenous injections of a pentobarbital sodium solution. The operation site was shaved and painted with iodine, and local anesthesia of 2% lidocaine with 1:80,000 epinephrine was then administered. The tibia was exposed through a longitudinal incision of about 4 cm. Using a dental bar, grinding of the tibia to the sizes described in the document was conducted. During drilling, the site was cooled with a physiological saline solution. The sample was embedded into the hole in the tibia (Fig. 1), and then the cut skin was sutured. The animals were killed 72 weeks after the operation.

The bone-embedded samples were excised after sacrifice. The samples were extirpated and divided into two pieces. One side of the tibia was allocated to TEM and immediately fixed in a 2.5% glutaraldehyde solution with a 0.2 M cacodylate buffer at 4 °C followed by demineralization in 5% EDTA (ethylene diamine tetracetic acid) solution for about 4 weeks at room temperature. The samples were removed and fixed for 1 h in 1% osmium tetroxide solution at room temperature, dehydrated, and embedded in EPON 812. Thin sections were prepared with Ultracut, double-stained with uranyl acetate and lead citrate by the authentic method, and examined with a JOEL 1200EX electron microscope (JOEL Ltd. Tokyo Japan) at 80 kV.

Others were fixed in a 10% formalin solution for light microscopy as follows: Samples were embedded in a polyester resin, and then sections were cut perpendicular to the interface of the samples and bone with a diamond cutter (crystal cutter and speed lap; Isomet (Buehler Ltd. USA)). Sections 50 μm in thickness were prepared and stained with toluidine blue.

3. Experiment Results

3.1 Results for Titanium

Titanium was surrounded by woven bone (immature bone) (Fig. 2), and fibrous tissues were observed partially at the interface (Fig. 3). TEM showed an

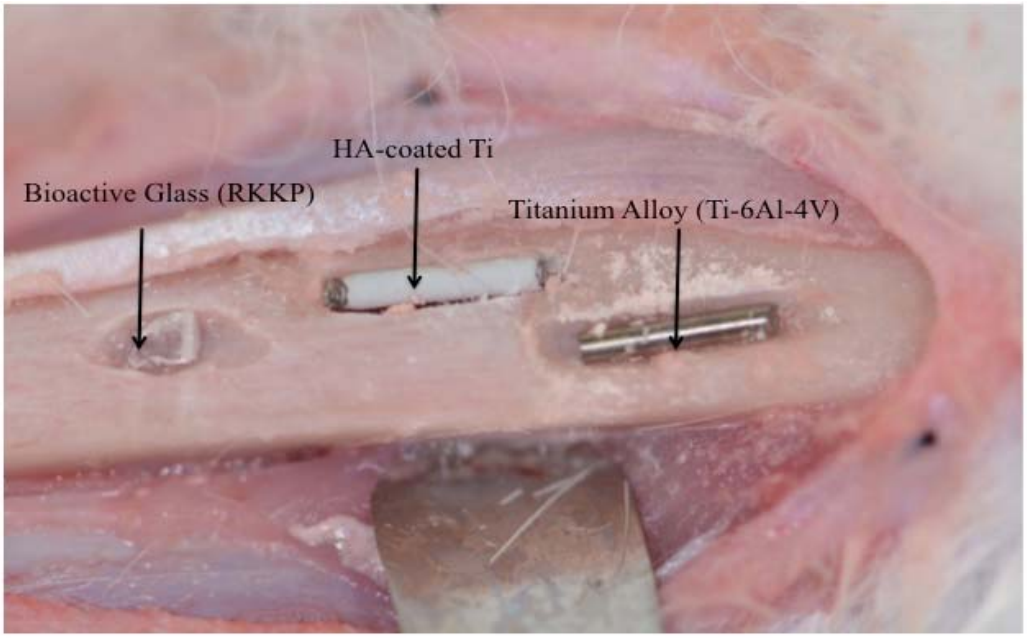

Fig. 1 We made a bone defect using a dental bar in a rabbit tibia and embedded the samples.

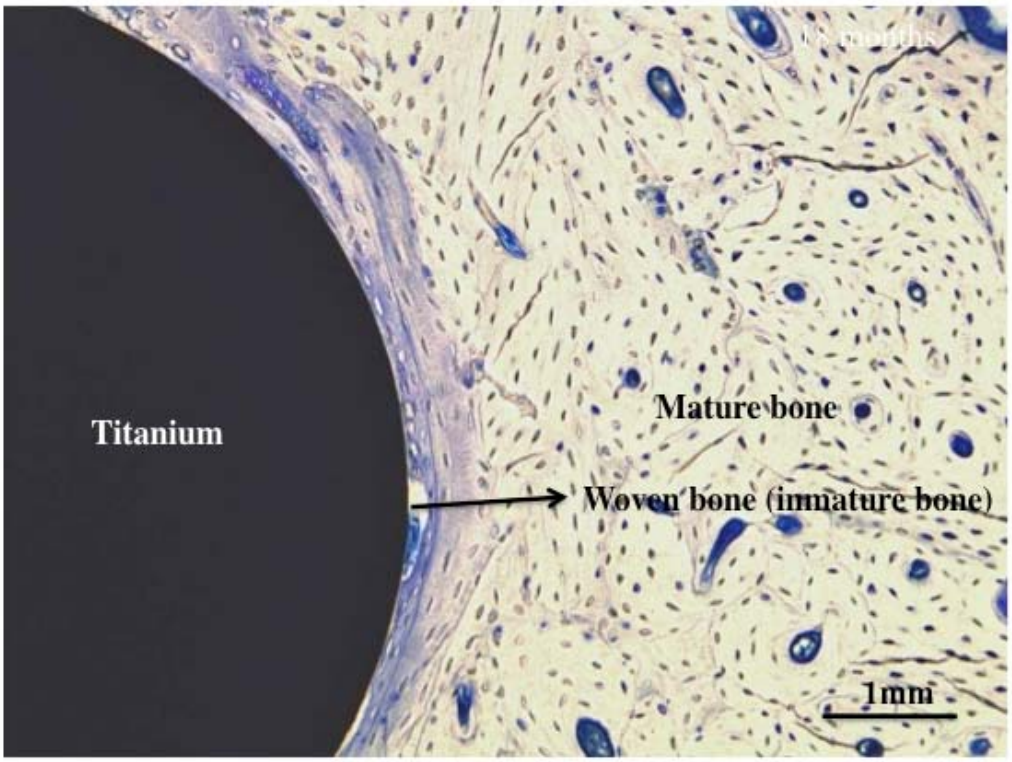

Fig. 2 Titanium (Ti-6Al-4V) was surrounded by woven bone (immature bone) (toluidine blue stain).

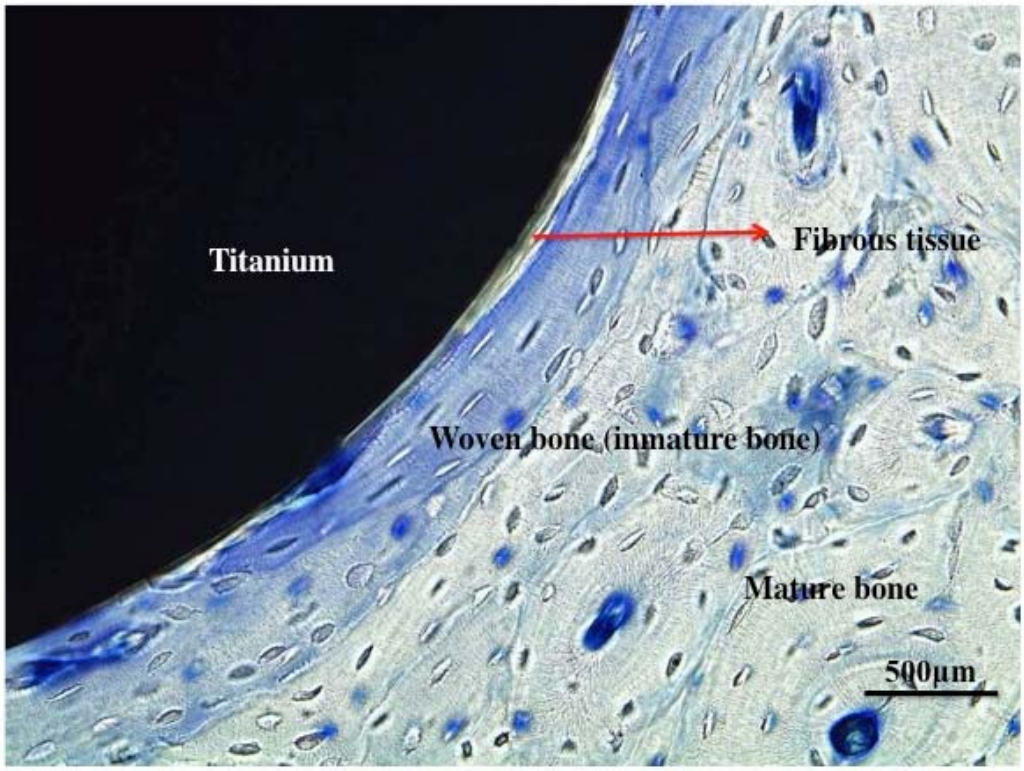

Fig. 3 Fibrous tissues can be seen partially at the interface (toluidine blue stain).

extremely thin-density layer responding to the titanium at the surface of the bone (Fig. 4).

3.2 Results for Coated HA

Although partially bonded woven bone (immature bone) was seen, the sample was mostly surrounded by mature bone. The dissolution of coated HA was observed to some degree (Fig. 5). TEM showed that HA bonded directly to the bone; however, it became partially separated, and some HA particles remaining in

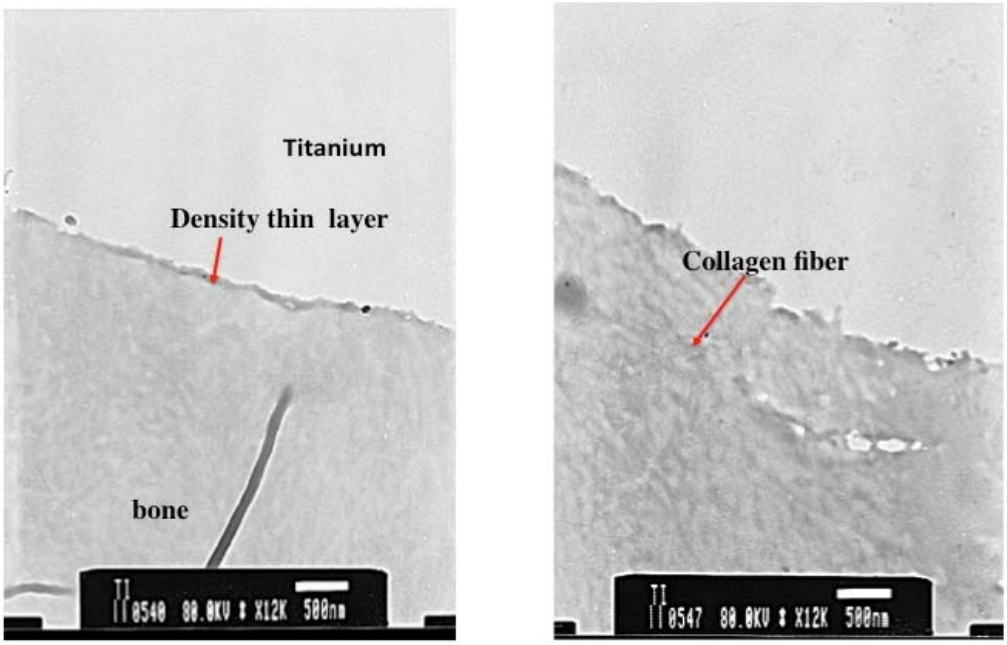

Fig. 4 TEM showed an extremely thin-density layer responding to the titanium at the surface of the bone.

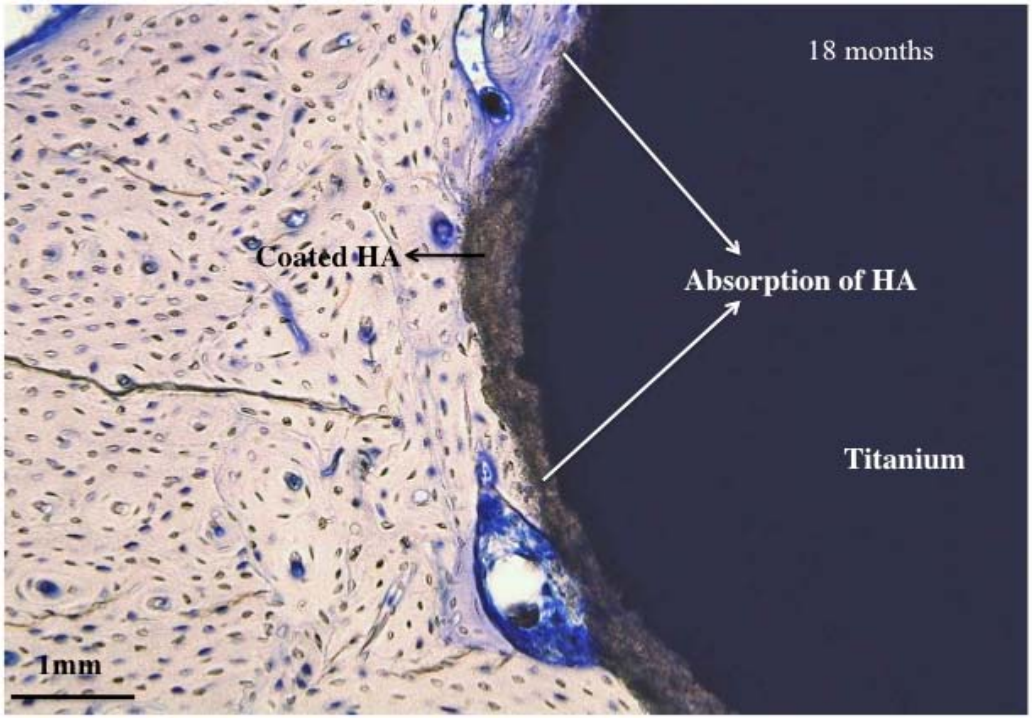

Fig. 5 In coated HA, although partially bonded woven bone (immature bone) was seen, the sample was mostly surrounded by mature bone. The dissolution of coated HA can be observed to some degree (toluidine blue stain).

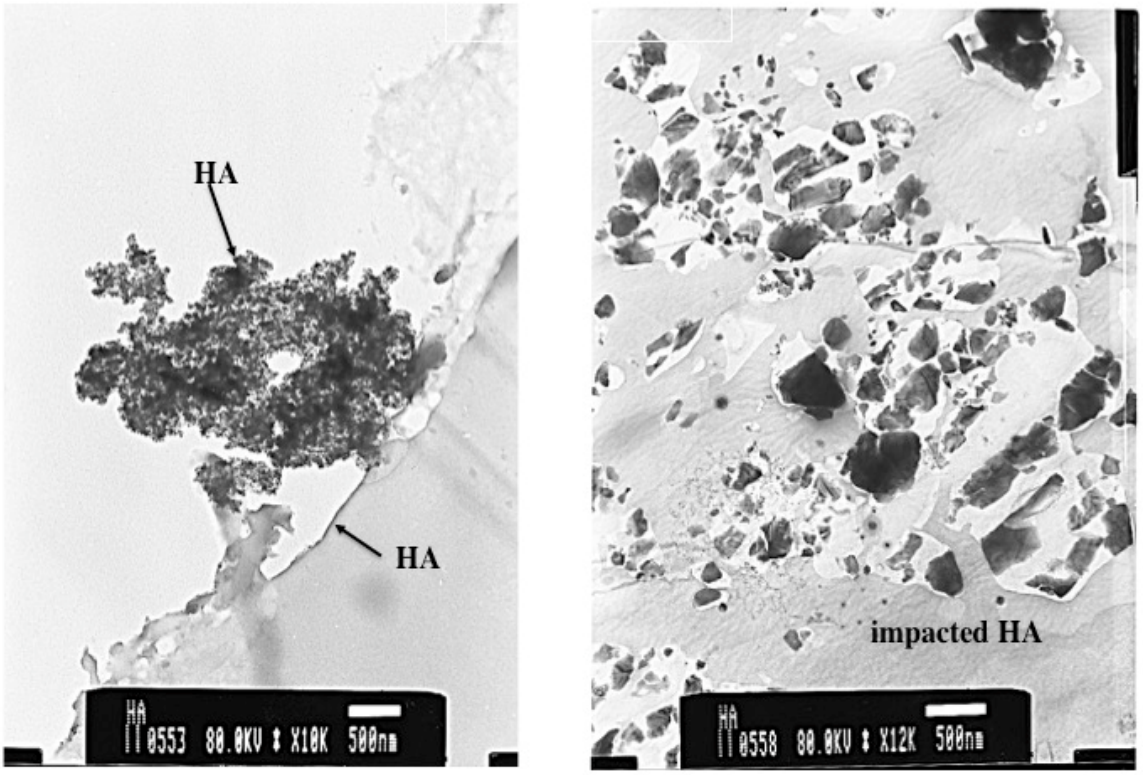

Fig. 6 TEM showed that HA bonded directly to the bone; however, it became partially separated, and some HA particles remaining in the bone can be seen.

the bone could be seen (Fig. 6).

3.3 Results for AP40

AP40 was surrounded by mature bone without inflammation (Fig. 7). TEM showed that AP40 bonded directly to the bone; however, we observed an amorphous layer between AP40 and the bone (Fig. 8).

3.4 Results for RKKP

RKKP was surrounded by mature bone without inflammation (Fig. 9). TEM showed that RKKP extends to the bone at the interface (Fig. 10).

4. Conclusion

We have developed and studied various biomaterials for approximately 40 years, with particularly focusing on the biocompatibility of biomaterials.

This time, based on *in vitro* results, we examined the biocompatibility of each using an optical microscope and TEM *in vivo*. As a result, at the optical level, interposition of fibrous tissues was observed in some titanium parts, and in coated HA, absorption images of HA were partially observed. These are believed to be due to the bioinert characteristics of titanium and the crystallinity of HA in coated HA. In contrast, in each novel bioactive glass (AP40, RKKP), an image of it directly binding to bones without any interposition of fibrous tissues was observed, leading us to believe that good biocompatibility with bones was shown. Furthermore, in TEM, RKKP directly contacted the bone cells without any interposition of an extracellular matrix or gel layer.

Krajewski et al. [13] compared the behavior of two bioactive silica-phosphate glasses, AP40 and RKKP, in a simulated biological environment. IR (infrared spectrum) and EDX (energy dispersive X-ray spectroscopy) analyses showed that the deposits formed on both glasses were composed of a calcium-deficient

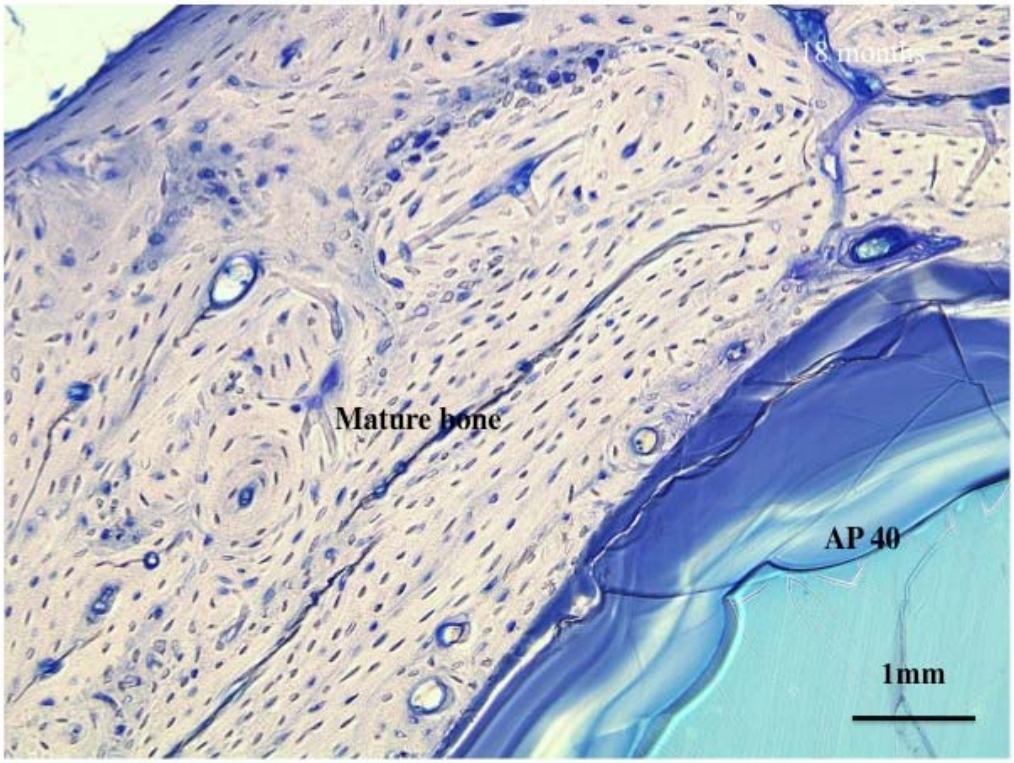

Fig. 7 AP40 was surrounded by mature bone without inflammation (toluidine blue stain).

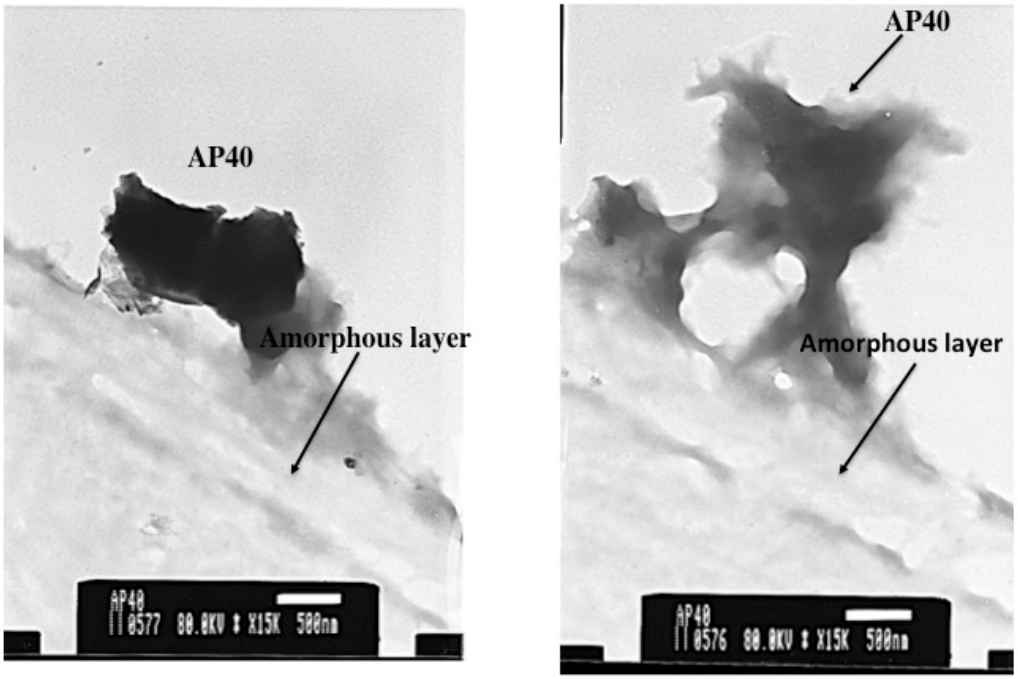

Fig. 8 TEM showed that AP40 bonded directly to the bone; however, we observed an amorphous layer between AP40 and the bone.

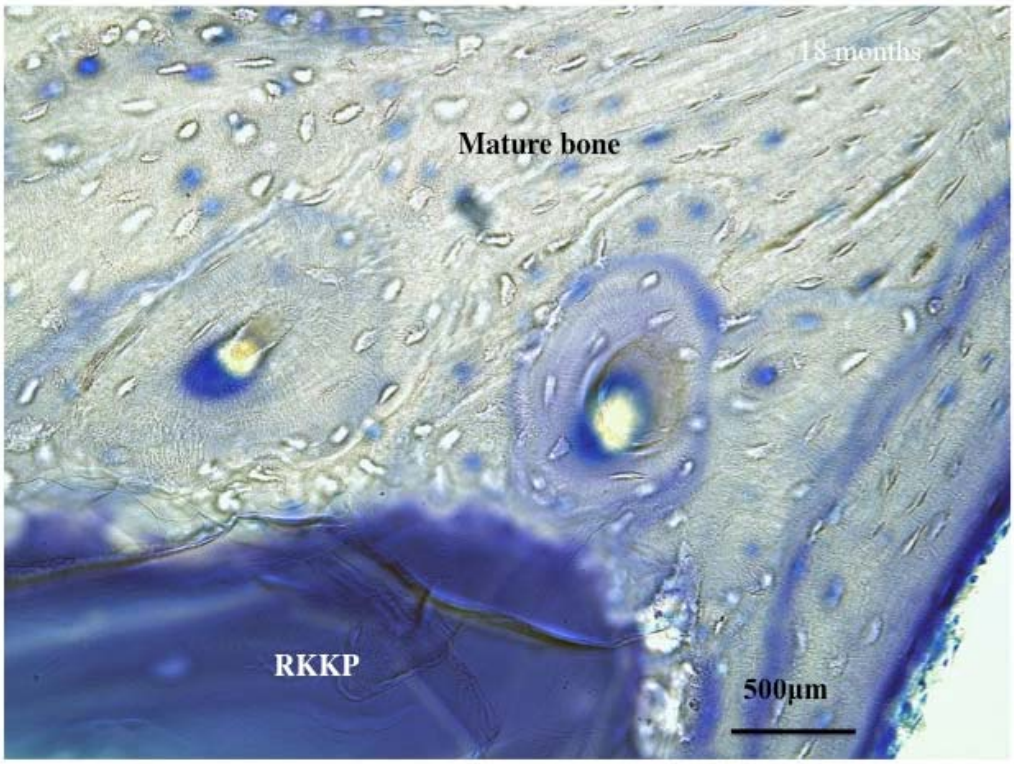

Fig. 9 RKKP was surrounded by mature bone, like AP40, without inflammation.

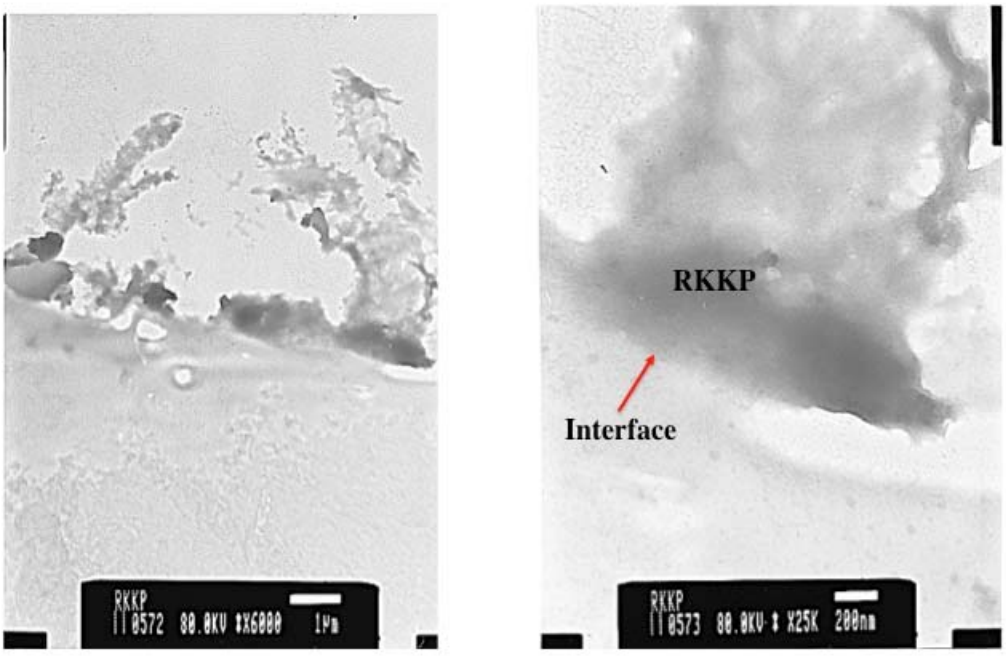

Fig. 10 TEM showed that RKKP extends to the bone at the interface (directly bound to the bone).

carbonate-apatite. However, the layer formed on the RKKP glass was found to be slightly more calcium-deficient and thinner than that on AP40. An EDX analysis revealed the presence of a small percentage of F-ions, but only in the layers formed on the RKKP. Fluorine ions are known to stabilize the apatitic lattice [14], and a small amount stimulates bone reconstruction (very small quantities of F-ions

enhance osteoblast proliferation) [15]. Thus, the relationship between bone and bioglass, including the *in vivo* and *in vitro* mechanisms, has been investigated in detail.

Bosetti et al. [17] reported that fibroblasts and osteoblast-like cells cultured on RKKP and AP40-coated zirconia showed a higher proliferation rate than, titanium leading to confluent cultures with a higher cell density and a generally better expression of osteoblast alkaline phosphatase activity than zirconia substrate. These results indicate that the surface chemical characteristics of AP40 and RKKP, which show similar properties, substantially enhance zirconia integration with bone cells (at least *in vitro*). Fini et al. [6] compared HA, Ti-6Al-4V, zirconia, alumina, AP40 and RKKP in a histomorphometric study using a rat model of osteopenia. The study did not identify which materials gave the best results; however, they proved the affinity of RKKP for osteopenia. In their study, only RKKP bounds directly to the cells without an intervening layer. These were the first TEM photographs in the world to show the relationship between bone cells and RKKP.

RKKP glass ceramics containing minor amounts of apatite crystals (8%) in a glassy matrix show good protein-binding capacities [5, 17]. Its non-isothermal crystallization behavior has been studied [9]. Small amounts of La_2O_3 and Ta_2O_5 were added to function as possible nuclei of deposition for the ions involved in bone formation. The presence of these oxides was shown to be able to modify the surface properties of the glass and influencing the protein absorption kinetics *in vitro* [16].

Regarding RKKP, many researchers have also reported on the properties and biocompatibility to bone tissue *in vivo* and *in vitro* [16-22]. Nicoli et al. [18] investigated the biocompatibility and osteointegration of zirconia (ZrO_2), either coated with RKKP bioglaze or uncoated, both *in vitro* and *in vivo*. Histomorphometry revealed that at 30 days, the affinity index was higher in the coated implants than in the uncoated ones, but the difference was not significant. Furthermore, Stanic et al. [19] evaluated the osteointegration of YSTZ (yttria-stabilized tetragonal zirconia), either coated with RKKP or uncoated in an animal model (Spraque Dawley rats) for 30 and 60 days. An *in vivo* histomorphometric evaluation revealed that at 30 days, the RKKP-coated YSTZ implants showed a significantly higher affinity index than the uncoated YSTZ implants. At 60 days, the coated implants behaved better than the controls, but the difference was not statistically significant.

These results are believed to further support the reports to date. Particularly, it is believed notable that RKKP also exhibited specificity to directly connect even cells of different origins *in vitro*.

From the above, novel bioactive glasses are considered to be biomaterials which can be fully applied in clinical practice as bone prosthetic materials, scaffolds for regenerative therapy, and coating materials for titanium, etc.

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