Journal of Minerals and Materials Characterization and Engineering, 2025, 13(5), 217-226
https://www.scirp.org/journal/jmmce
ISSN Online: 2327-4085
ISSN Print: 2327-4077

Enhancement of Calcium Phosphate Crystal Formation and Bone-Bonding Function on Biological Bone Surfaces by Low-Intensity Pulsed Ultrasound (LIPUS) Irradiation

Masanori Kobayashi

Department of Physical Therapy, College of Life and Health Sciences, Chubu University, Kasugai, Japan
Email: chubukoba@fsc.chubu.ac.jp

How to cite this paper: Kobayashi, M. (2025) Enhancement of Calcium Phosphate Crystal Formation and Bone-Bonding Function on Biological Bone Surfaces by Low-Intensity Pulsed Ultrasound (LIPUS) Irradiation. *Journal of Minerals and Materials Characterization and Engineering*, **13**, 217-226.
https://doi.org/10.4236/jmmce.2025.135013

Received: June 23, 2025
Accepted: August 17, 2025
Published: August 20, 2025

Abstract

The excellent osteoconductive properties of hydroxyapatite materials are well known, and hydroxyapatite-coated prostheses and dental implants for osseointegration are widely used in clinical practice. However, in clinical practice, there is a demand for even faster integration between implants and living bone from the perspective of rehabilitation and postoperative recovery of Activities of Daily Living, and research is progressing to achieve earlier osseointegration. Against these backgrounds, the author focused on Low-Intensity Pulsed Ultrasound (LIPUS), and has already reported that LIPUS stimulation promotes osseointegration through a physical crystallization mechanism of bone-like calcium phosphate-apatite deposited on the surface of hydroxyapatite material. This study examined whether Low-Intensity Pulsed Ultrasound (LIPUS) can promote the physical crystallization of bone-like calcium phosphate on natural bone surfaces. Natural bone specimens from porcine femurs were immersed in simulated body fluid and sonicated with LIPUS. The surface precipitate conditions were analyzed using scanning electron microscopy and X-ray diffraction. The results showed significantly more calcium phosphate precipitation on the surfaces of the samples irradiated by LIPUS than on the surfaces of the non-irradiated samples. These results suggest that ultrasound irradiation promotes the precipitation of bone-like hydroxyapatite on biological bone and realizes the excellent osseointegration of the hydroxyapatite material surface with the biological bone matrix.

Keywords

Calcium Phosphate Crystallization, Simulated Body Fluid (SBF), Biological Bone, Low-Intensity Pulsed Ultrasound (LIPUS), Osseointegration

1. Introduction

Among bioactive materials, Hydroxyapatite (HA) exhibits excellent biocompatibility and is known to demonstrate Osteoconductivity, promoting the new formation and growth of surrounding bone within the body, and Osseointegration, direct bonding with bone. Currently, utilizing these properties, the development of medical implants such as artificial joints, dental implants, and artificial bones with hydroxyapatite coating on their surfaces is actively being pursued. The aim is to achieve early and strong bonding with natural living bon [1]-[3]. However, in clinical practice, there is a demand for even faster integration between implants and living bone from the perspective of rehabilitation and postoperative recovery of Activities of Daily Living (ADL), and research is progressing to achieve earlier osseointegration.

Against these backgrounds, the author focused on Low-Intensity Pulsed Ultrasound (LIPUS), which is used in bone fracture treatment, and have reported that the ultrasound irradiation to bioactive material such as bioactive titanium and hydroxyapatite promotes the amount of bone-like hydroxyapatite crystal deposition on the material surface, leading to early bonding between the material and natural living bone via this HA layer in past in vitro and *in vivo* studies [4]-[7]. This ultrasound-induced enhancement of osseointegration suggests the involvement of a crystal-chemical crystallography mechanism regarding the precipitation and growth enhancement of bone-like calcium phosphate crystals, which is different from biological mechanisms such as osteogenesis. The possibility of a stable implantation and attachment between dental implants and living bone, as well as early achievement of osseointegration, is expected.

In a previous study here, the author reported that the crystallographic orientation of the hydroxyapatite sample surface limits the initial bone-like calcium phosphate crystal structure induced by this LIPUS [8]. In this study, biological bone was selected as an additional hydroxyapatite sample with different surface structures, and the crystal growth of bone-like calcium phosphate crystals on the surface of the biological bone under LIPUS was observed and compared with the results of a previous study using artificial hydroxyapatite.

2. Materials and Methods

In this study, as a method to evaluate the effect of collagen fibers on apatite precipitation under LIPUS irradiation, natural bone specimens immersed in a pseudo-body fluid (SBF) without cellular components were subjected to ultrasound irradiation as in the previously reported simulation experimental method [8], the changes in calcium phosphate apatite deposition on the specimen surface were observed and compared.

2.1. Experimental Materials

In this experiment, living bone from meat pigs was used as a biological specimen. The bone was harvested immediately after slaughter and frozen to preserve its con-

dition. This biological specimen needs to be a type of hydroxyapatite material, allowing for comparison with previous data obtained using synthetic hydroxyapatite. Cortical bone was excised from the femur, and after thawing, soft tissues such as the periosteum were removed. The specimen was then heat-treated in a muffle furnace at 200˚C to eliminate residual soft tissues, including internal collagen, following the method described by Sugita *et al.* for collagen removal through thermal processing [9].

After that, specimen surfaces were sequentially polished with emery paper #300 to #1500 to adjust the surface roughness. From the processed bone specimens described above, samples with dimensions of 30 mm × 20 mm × 5 mm were sectioned.

Figure 1 shows SEM images of the surface of the biological bone specimen used.

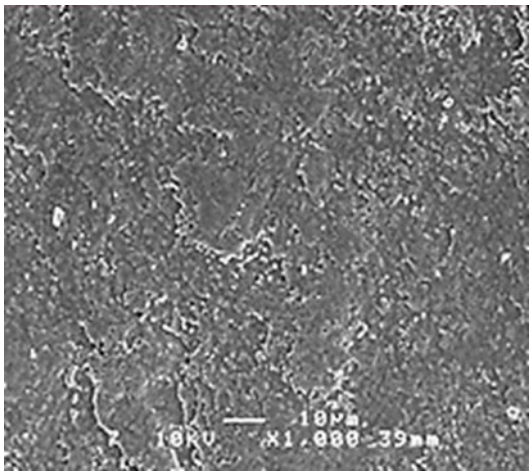

Figure 1. The SEM image of natural bone specimen surface in this study (×1000).

2.2. Simulated Body Fluid (SBF) Soaking Test

As a basic osseointegration test, the simulated body fluid (SBF) soaking method was performed according to Kokubo's study (10) in order to evaluate the apatite-forming ability of bioactive materials, in the same manner as the previous study. Hank's balanced solution (Lonza®; USA) was used as an SBF solution, and maintained at pH over 7.0 and 37˚C and replaced every two days. Ultrasound radiation was applied by using Sonic Accelerated Fracture Healing System (SAFHS; Smith & Nephew, Memphis, TN, USA; Teijin Pharma, Tokyo, Japan). The treatment head module delivered ultrasound waves with 1.5 MHz, 200 μsec signal term, and spatial average intensity of 30 mW/cm^2. A total of 20 natural bone samples were soaked in SBF and subjected to ultrasound stimulation for 20 min daily during the operation term for three days, one week, and two weeks, respectively. As a control, the same specimens were left in SBF without ultrasound radiation under the same experimental conditions (Figure 2).

Five or seven specimens were removed from the SBF under the above conditions after a fixed immersion period (3 days, 1 week, 2 weeks), and the state of bone-like precipitates on the surface of the biological bone specimens was observed by

Scanning Electron Microscopy (SEM) and X-Ray Diffraction (XRD). These experimental methods are the same as those used in the previous report [8].

The entire series of simulation experiments described above was repeated three times.

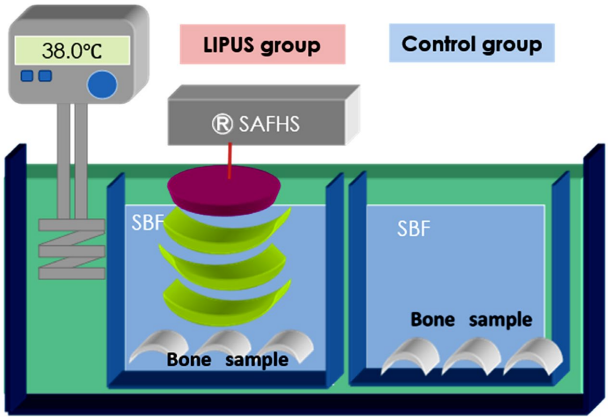

Figure 2. Diagram of the pulsed ultrasound wave radiation on natural bone specimens in Simulated Body Fluid (SBF).

3. Results

3.1. SEM Images of the Sample Surfaces after Up to 2 Weeks of Immersion in SBF

Figure 3 shows a comparison of the results of SEM observations of the surfaces of specimens in the ultrasonic-irradiated group and the non-irradiated group (control group) after up to 2 weeks of immersion in SBF.

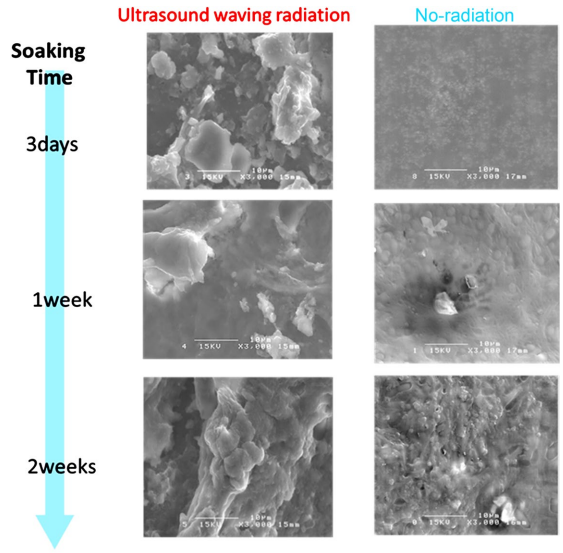

Figure 3. SEM micrographs of the surfaces of the natural bone specimens of the LIPUS and control groups (×3000).

In both groups, an increase in precipitates was observed with longer immersion

time in SBF; however, crystal precipitation was clearly more pronounced in the LIPUS-irradiated group. The morphology of the precipitates in both groups appeared as irregular, stacked polygonal crystals, which differed significantly from the precipitate morphology previously reported on artificial hydroxyapatite surfaces [8].

3.2. X-Ray Diffraction (XRD)

The deposits on the specimen surface in LIPUS group after 3 days of immersion in SBF were identified by X-Ray Diffraction (XRD). The XRD peak patterns are shown in Figure 4. The XRD of the deposits showed a broad diffraction pattern, and no clear diffraction peaks were observed. However, some diffraction peaks attributable to Hydroxyapatite (HA) were partially observed. Although there are concerns about overlapping with diffraction lines from the living bone specimen surface (9), which is the base matrix, because of the small amount of deposits, the presence of diffraction peaks characteristic of HA suggests that the deposits are calcium phosphate with an imperfect HA crystal structure.

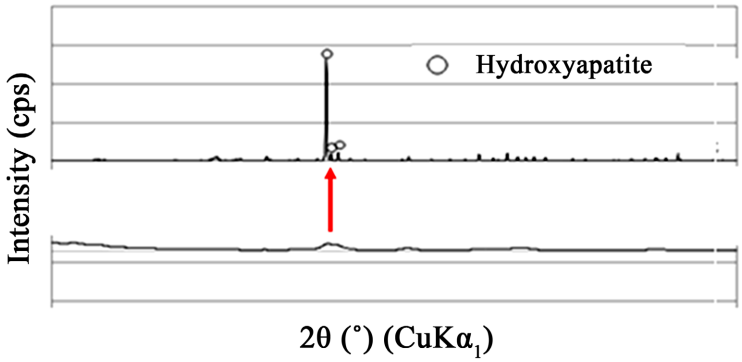

Figure 4. XRD of the LIPUS group specimen surface after soaking in SBF for three days.

3.3. Measurement of the Mass of Ca-P Crystallization

The amount of calcium phosphate crystal deposition on the surface was quantified by measuring the weight difference of the specimens before and after immersion in SBF.

Figure 5 shows the measured mass change as hydroxyapatite-like precipitation (crystalline calcium phosphate) on the surfaces of the LIPUS group and control group after SBF soaking.

In both groups, the amount of Ca-P precipitation increased with longer SBF soaking times. However, the specimens in the LIPUS group exhibited a markedly greater amount of precipitation compared to those in the control group.

4. Discussion

The author has previously reported the initial promotion effect of bone-like apatite layer formation by LIPUS irradiation as a method to enhance osseointegration on the surface of bioactive titanium and hydroxyapatite [4]-[7].

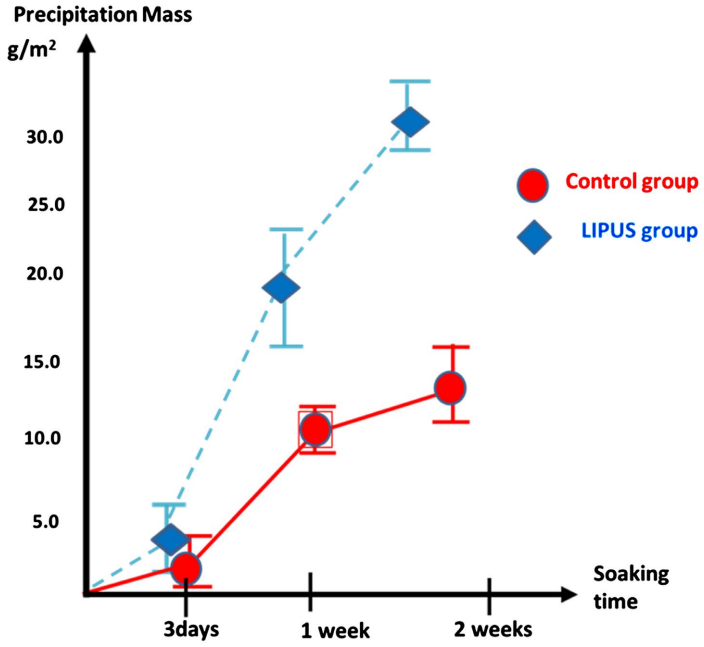

Figure 5. The soaking time dependence of mass of P/Ca precipitation formation on the specimen's surfaces in the LIPUS and control groups.

The mechanism of apatite precipitation on the surface of bioactive materials from Simulated Body Fluid (SBF), which mimics the *in vivo* reaction, has been explained by the physical deposition and crystal nucleation process of ions such as PO_4^{2-} and Ca^{2+} from SBF onto the material surface [10]. This apatite precipitation process is a purely physicochemical change without any biological action by osteoblast cells, etc. It is thought that the mechanism by which LIPUS enhanced this calcium phosphate crystal growth is due to the amplification of PO_4^{2-} and Ca^{2+} ion concentrations and thermal fluctuations in SBF by appropriate ultrasonic micro-waving, which promoted nucleation on the bioactive material surface. Furthermore, it is inferred that the circulation and stirring by ultrasound also effectively accelerated crystal growth.

In the case of the natural bone apatite sample, it was confirmed that calcium phosphate crystal growth in bone matrix apatite increased over time, which is more than has been reported in the past for biomaterials. It was also confirmed that LIPUS irradiation promoted apatite crystal growth in biological bone. The mechanism by which calcium phosphate crystals grow strongly in this biological bone with LIPUS irradiation is considered to be almost the same as the mechanism described above.

Compared to the two types of synthetic biomaterial hydroxyapatite used in my previous study [8], the biological bone specimens in the present experiment exhibited a significantly greater growth of calcium phosphate crystals, as shown by the quantitative measurement of the precipitates in Figure 5. To compare the surface conditions between the present and previously reported specimens, SEM images of the LIPUS-irradiated group are shown comparatively in Figure 6.

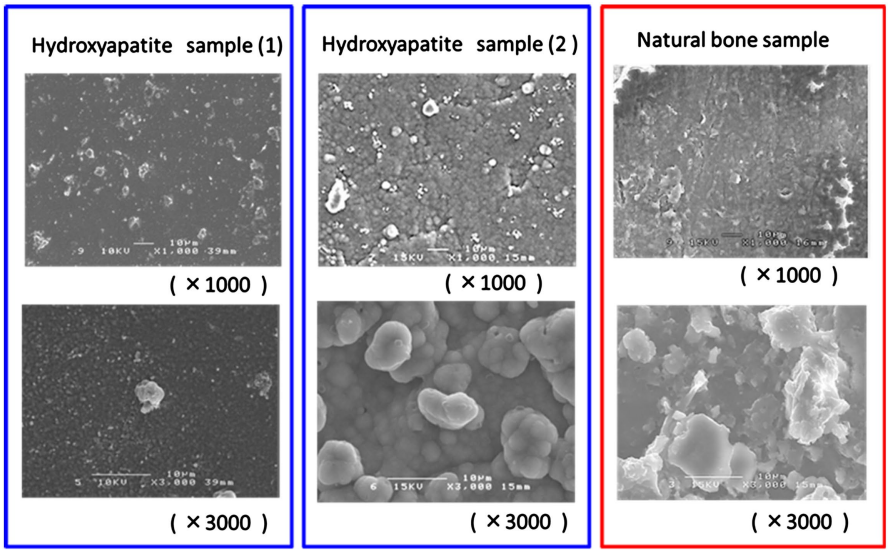

Figure 6. Comparison of SEM images of the surface of samples (hydroxyapatite and natural bone) of LIPUS group soaked in SBF for 3 days.

While the surface of artificial apatite showed relatively slow and stable crystallization with granular crystal morphology, the biological bone sample demonstrated more active crystal growth characterized by spindle-shaped protrusions.

These results are presumed to be due to differences in the surface structure of the apatite specimens, as previously reported. Regarding the enhancement of calcium phosphate crystal formation induced by LIPUS, epitaxial factors related to the crystal orientation of the hydroxyapatite surface were found to regulate the growth of initially nucleated, bone-like calcium phosphate. The synthetic hydroxyapatite used in the previous study, in particular, had a dense structure and a surface crystal orientation that was almost exclusively aligned along the c-axis of apatite. This also limited the amount of crystal deposition.

On the other hand, it is known that biological bone exhibits a broad XRD pattern, as reported in previous studies on biological bone [11], indicating a considerable variation in the crystal orientation of apatite on the bone matrix surface. This is attributed to the fact that bone is inherently a composite material consisting of apatite within the bone matrix and collagen tissue, and calcium phosphate tends to deposit and grow on the polymeric collagen molecular chains, which have an amorphous nature. As a result, apatite crystals with multiple orientations are likely to form. Such a surface condition provides a high degree of freedom in nucleation orientation for calcium phosphate and is considered to offer a favorable environment for crystal growth from SBF. Furthermore, the crystallization process is presumed to be enhanced by LIPUS. This is also suggested by the XRD pattern of the calcium phosphate precipitates shown in **Figure 4**.

Of course, the present study was a simulation experiment using bone matrix of porcine femur, and compared to the actual *in vivo* environment, several situations that differ from the bio-physiological conditions and limitations in the reproduc-

ibility of the results are possible. Especially in the post-operative period after the replacement of actual implant materials, a biological response may be a partial inflammation, which may lead to an increase in bioactive substances and temporary changes in ion concentrations and pH of body fluids, that may significantly affect the nucleation and growth of apatite crystals on the surface of the material. However, such biological responses are thought to occur up to 1 - 2 weeks postoperatively, and previous reports on the evaluation of bioactivity of biomaterials using SBFs have demonstrated a correlation between the osteoconductive function of materials actually used in clinical practice and the state of apatite formation within the SBF [12] [13]. Therefore, although accurate prediction is difficult, it is considered reliable to evaluate the bond-promoting function of LIPUS with implant materials via the promotion of apatite formation on the bio-bone surface by the present experimental method.

On the other hand, as for clinical field, there have already been clinical reports on the application of LIPUS for implant placement in dentistry, and it has been confirmed that LIPUS promotes bonding between hydroxyapatite-coated implants and bone [14]-[16]. However, all of these cases were several months after surgery, and the effect of LIPUS is also attributed to biological factors, such as the mechanical stimulation to the osteocytes and fibroblasts, which promoted osteogenesis, and the effects of biomineralization, such as nucleation and crystal growth of bone-like apatite on the material surface prior to biological reactions as described here, are not mentioned. Also, in the field of basic research, to date, there is no study other than ours that has reported the effect of LIPUS on the promotion of osseointegration, focusing on micro-level observations of the promotion of calcium phosphate crystals on the surface of osteoconductive materials. It's impossible to directly observe the state of implant materials in humans during the early postoperative period. While we've tried to gather *in vivo* data through animal experiments, there's still variability in surgical techniques for fixing implants to bone, in addition to the many biological reactions that are tough to visualize with SEM. However, our previous Energy-Dispersive X-ray spectroscopy (EDS) analysis clearly showed vigorous calcium phosphate deposition on material surfaces when exposed to LIPUS (7).

In this experiment, porcine bone was used instead of human bone, which may be a potential difference from human bone, but since the XRD pattern of the measured samples resembles the crystal structure of apatite in human bone, it is assumed that bone-like hydroxyapatite crystallization in the actual human bone matrix is also amplified by LIPUS as well as this results. This suggests that LIPUS irradiation around HA-coated implants can enhance calcium phosphate nucleation and crystal growth not only on the HA implant surface but also on the surrounding bone matrix side, resulting in earlier and more stable bonding (osseointegration) at the contact zone between the implant and bone matrix.

5. Conclusion

This study suggested that the clinical application of LIPUS irradiation has great

potential for the enhancement of osseointegration or bone attachment of HA implants through the promotion of nucleation and crystal formation of bone-like Calcium phosphate on biological bone surfaces. Furthermore, in order to get more detail information regarding clinical application for the enhancement of osseointegration by the ultrasound wave irradiation, further study concerning the apatite formation on the crystallographic characteristics of HA surfaces would be necessary.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

References

[1] Inagaki, M. and Kameyama, T. (2007) Plasma Biomaterial Coating. *Journal of Plasma and Fusion Research*, **83**, 595-560. (In Japanese)

[2] Thomas, K.A., Kay, J.F., Cook, S.D. and Jarcho, M. (1987) The Effect of Surface Macrotexture and Hydroxylapatite Coating on the Mechanical Strengths and Histologic Profiles of Titanium Implant Materials. *Journal of Biomedical Materials Research*, **21**, 1395-1414. https://doi.org/10.1002/jbm.820211205

[3] De Andrade, M.C., Sader, M.S., Filgueiras, M.R.T. and Ogasawara, T. (2000) Microstructure of Ceramic Coating on Titanium Surface as a Result of Hydrothermal Treatment. *Journal of Materials Science*: *Materials in Medicine*, **11**, 751-755. https://doi.org/10.1023/a:1008984030540

[4] Kobayashi, M., Noda, K. and Tatematsu, N. (2010) Preliminary in Vitro Study on Enhancement of Bone-Like Hydroxyapatite Formation on Bio-Active Titanium Alloy by Low-Intensity Pulsed Ultrasound Waving for Early Bone Bonding. *Journal of Biomechanical Science and Engineering*, **5**, 449-460. https://doi.org/10.1299/jbse.5.449

[5] Kobayahsi, M. (2017) Study on Enhancement of Osseointegration of the Bio-Active Titanium Implant by Low Intensive Ultrasound Wavefull Text. *International Journal of Biomedical Engineering and Science*, **4**, 1-10. https://doi.org/10.5121/ijbes.2017.4401

[6] Kobayahsi, M., Shiraishi, M. and Kinoshita, M. (2018) Study on Enhancement of Osseointegration of the Bio-Active Titanium Implant by Low Intensive Ultrasound Wave. *International Journal of Biomedical Engineering and Science*, **5**, 1-10. https://doi.org/10.5121/ijbes.2018.5101

[7] Kobayashi, M. (2020) Enhancement of Osseointegration of Hydroxyapatite Implants by Low-Intensity Ultrasound Irradiation. *Journal of Osseointegration*, **12**, 1-8.

[8] Kobayashi, M. (2021) The Effect of Material Surface Microstructure on the Enhancement of Bone-Bonding Ability of a Hydroxyapatite-Implant by Low-Intensity Pulsed Ultrasound (LIPUS). *Materials Sciences and Applications*, **12**, 42-51. https://doi.org/10.4236/msa.2021.121003

[9] Sugita, K., Shirai, K., Wada, K. and Kawamura, A. (1977) Changes of Collagen in Foods during Heating (Part I. Solubilization of Pig Skin Collagen). *Nippon Shokuhin Kogyo Gakkaishi*, **24**, 311-315. https://doi.org/10.3136/nskkk1962.24.311

[10] Kokubo, T. and Takadama, H. (2006) How Useful Is SBF in Predicting *in Vivo* Bone Bioactivity? *Biomaterials*, **27**, 2907-2915. https://doi.org/10.1016/j.biomaterials.2006.01.017

[11] Tanaka, S. and Machinaga, O. (2005) Similarity of Crystal Structure between Hy-

droxy-Apatite and Its Precursor Crystal. Report of the Research Institute of Industrial technology, Nihon University, 35-39. (In Japanese)

[12] Shin, K., Acri, T., Geary, S. and Salem, A.K. (2017) Biomimetic Mineralization of Biomaterials Using Simulated Body Fluids for Bone Tissue Engineering and Regenerative Medicine. *Tissue Engineering Part A*, **23**, 1169-1180. https://doi.org/10.1089/ten.tea.2016.0556

[13] Baino, F. and Yamaguchi, S. (2020) The Use of Simulated Body Fluid (SBF) for Assessing Materials Bioactivity in the Context of Tissue Engineering: Review and Challenges. *Biomimetics*, **5**, Article 57. https://doi.org/10.3390/biomimetics5040057

[14] Zhou, H., Hou, Y., Zhu, Z., Xiao, W., Xu, Q., Li, L., *et al.* (2016) Effects of Low-intensity Pulsed Ultrasound on Implant Osseointegration in Ovariectomized Rats. *Journal of Ultrasound in Medicine*, **35**, 747-754. https://doi.org/10.7863/ultra.15.01083

[15] Abdulhameed, E.A., Enezei, H.H., Omar, M., Komori, A., Sugita, Y., Hegazy, F.A., *et al.* (2017) The Effect of Low Intensity Pulsed Ultrasound Therapy on Osseointegration and Marginal Bone Loss around Dental Implants. *Journal of Hard Tissue Biology*, **26**, 323-330. https://doi.org/10.2485/jhtb.26.323

[16] Rego, E.B., Takata, T., Tanne, K. and Tanaka, E. (2012) Current Status of Low Intensity Pulsed Ultrasound for Dental Purposes. *The Open Dentistry Journal*, **6**, 220-225. https://doi.org/10.2174/1874210601206010220