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Biomaterials 27 (2006) 2907–2915
Biomaterials
www.elsevier.com/locate/biomaterials

Leading Opinion

How useful is SBF in predicting in vivo bone bioactivity? ☆

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Received 6 September 2005; accepted 13 January 2006
Available online 31 January 2006

Abstract

The bone-bonding ability of a material is often evaluated by examining the ability of apatite to form on its surface in a simulated body fluid (SBF) with ion concentrations nearly equal to those of human blood plasma. However, the validity of this method for evaluating bone-bonding ability has not been assessed systematically. Here, the history of SBF, correlation of the ability of apatite to form on various materials in SBF with their in vivo bone bioactivities, and some examples of the development of novel bioactive materials based on apatite formation in SBF are reviewed. It was concluded that examination of apatite formation on a material in SBF is useful for predicting the in vivo bone bioactivity of a material, and the number of animals used in and the duration of animal experiments can be reduced remarkably by using this method.

Keywords: Bioactivity; Bone; Hydroxyapatite; In vitro test; Osteoconduction; SBF

1. Introduction

Artificial materials implanted into bone defects are generally encapsulated by a fibrous tissue, leading to their isolation from the surrounding bone. However, in 1972, Hench et al. showed that some glasses in the Na_2O–CaO–SiO_2–P_2O_5 system, called Bioglass, spontaneously bond to living bone without the formation of surrounding fibrous tissue [1]. Since then, several types of ceramic, such as sintered hydroxyapatite [2], sintered β-tricalcium phosphate [3], apatite/β-tricalcium phosphate biphasic ceramics [4], and glass-ceramic A–W containing crystalline apatite and wollastonite [5] have been also shown to bond to living bone, and they are used clinically as important bone substitutes. However, these ceramics are not compatible mechanically to the surrounding bone. The development of bone-bonding materials with different mechanical properties is desired.

This desire leads to two questions: what type of material bonds to living bone; and are animal experiments the only one way to test for bone bonding, that is, to identify a material with in vivo bone bioactivity? In 1991, we proposed that the essential requirement for an artificial material to bond to living bone is the formation of bonelike apatite on its surface when implanted in the living body, and that this in vivo apatite formation can be reproduced in a simulated body fluid (SBF) with ion concentrations nearly equal to those of human blood plasma [6]. This means that the in vivo bone bioactivity of a material can be predicted from the apatite formation on its surface in SBF. Since then, in vivo bone bioactivity of various types of materials have been evaluated by apatite formation in SBF. However, the validity of this method has not been systematically assessed.

Here, the history of SBF, correlation of the ability of apatite to form on various materials in SBF with their in vivo bone bioactivities, and some examples of successful development of novel bioactive materials based on the apatite formation on their surfaces in SBF are reviewed.

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doi:10.1016/j.biomaterials.2006.01.017

2. History of SBF

In 1980, Hench et al. showed that a SiO_2-rich layer and calcium phosphate film form on the surface of Bioglass when implanted in the body environment, which allows bonding to living bone, and that the in vivo formation of the calcium phosphate film can be reproduced in a buffer solution consisting of Tris hydroxymethylaminomethane and hydrochloric acid (Tris buffer solution) at pH 7.4 [7].

On the other hand, Kitsugi et al. showed that the SiO_2-rich layer does not form on glass-ceramic A–W, but a calcium phosphate layer forms on its surface in the living body, allowing bonding to living bone [8]. Subsequently, Kokubo et al., using micro X-ray diffraction, identified this calcium phosphate layer as crystalline apatite [9]. In addition, in 1990, they showed that the in vivo apatite formation on the surface of glass-ceramic A–W can be reproduced in an acellular SBF with ion concentrations nearly equal to those of the human blood plasma, but not in a Tris buffer solution [10,11]. Kokubo et al. [10] and Hench et al. [12] also independently confirmed the formation of apatite on the surface of Bioglass 45S5-type glass in SBF.

Detailed analysis of the surface apatite formed in SBF, by means of thin film X-ray diffraction (TF-XRD), Fourier transform infrared spectroscopy, scanning electron microscopy and transmission electron microscopy, showed that it was similar to bone mineral in its composition and structure [10,11,13]. As a result, it was speculated that osteoblasts might preferentially proliferate and differentiate to produce apatite and collagen on its surface. Thus formed apatite might bond to the surface apatite as well as to the surrounding bone. Consequently, a tight chemical bond is formed between the material and the living bone through the apatite layer. In contrast, glass-ceramic A–W (Al), which also contains apatite and wollastonite, but in a glassy matrix containing Al_2O_3, and hence does not bond to living bone, did not have apatite form on its surface, both in vivo and in SBF [11,14]. Based on these results, in 1991 it was proposed that the essential requirement for a material to bond to living bone is the formation of bonelike apatite on its surface in the living body and that this in vivo apatite formation can be reproduced in SBF. This means that the in vivo bone bioactivity of a material can be predicted by examining apatite formation on its surface in SBF [6].

It should be noted here that the original SBF used by Kokubo et al. [10] and Hench et al. [12] lacks the SO_4^{2+} ions contained in human blood plasma [15], as shown in Table 1. This was corrected in papers [6,16] published by Kokubo et al. in 1991. Since then, the corrected SBF has been used as "SBF" by many researchers.

It should be also noted here that SBF is a solution highly supersaturated with respect to apatite [17]. It is not easy to prepare clear SBF with no precipitation. Therefore, a detailed recipe for preparation of SBF was reported in 1995 by Cho et al. [18].

However, it can be seen from Table 1 that corrected SBF is still richer in Cl^- ion and poorer in HCO_3^- ion than human blood plasma. In 2003, Oyane et al. tried to correct this difference [19] by preparing a revised SBF (r-SBF) in which the concentrations of Cl^- and HCO_3^- ions were, decreased and increased respectively, to the levels of human blood plasma. However, calcium carbonate has a strong tendency to precipitate from this SBF, as it is supersaturated with respect to not only apatite, but also calcite [20]. In 2004, Takadama et al. proposed a newly improved SBF (n-SBF) in which they decreased only the Cl^- ion concentration to the level of human blood plasma, leaving the HCO_3^- ion concentration equal to that of the corrected SBF (c-SBF) [21]. This improved SBF was compared with the corrected, i.e., conventional, c-SBF in its stability and the reproducibility of apatite formation on synthetic materials. Both SBFs were subjected to round robin testing in ten research institutes. As a result, it was confirmed that the c-SBF does not differ from n-SBF in stability and reproducibility [21]. Through this round robin testing, the method for preparing c-SBF was carefully checked and refined so that the SBF could be easily prepared. This refined recipe for preparing SBF is given in Appendix A of this paper, accompanied with procedure of apatite-forming ability test.

In 2003, conventional SBF with the refined recipe was proposed to the Technical Committee ISO/TC150 of International Organization for Standardization as a solution for in vitro measurement of apatite-forming ability of implant materials and is being discussed by the committee.

Table 1
Ion concentrations of SBFs and human blood plasma

	Ion concentration (mM)							
	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}
Human blood plasma [15]	142.0	5.0	1.5	2.5	103.0	27.0	1.0	0.5
Original SBF	142.0	5.0	1.5	2.5	148.8	4.2	1.0	0
Corrected SBF (c-SBF)	142.0	5.0	1.5	2.5	147.8	4.2	1.0	0.5
Revised SBF (r-SBF)	142.0	5.0	1.5	2.5	103.0	27.0	1.0	0.5
Newly improved SBF (n-SBF)	142.0	5.0	1.5	2.5	103.0	4.2	1.0	0.5

3. Qualitative correlation of apatite formation in SBF with in vivo bone bioactivity

As described above, a glass in the Na_2O–CaO–SiO_2–P_2O_5 system named Bioglass 45S5 has apatite form on its surface in SBF [10]. This glass was confirmed to bond to living bone through a calcium phosphate layer [7]. Glasses in the Na_2O–CaO–B_2O_3–Al_2O_3–SiO_2–P_2O_5 system were also found to have a calcium phosphate layer form on their surfaces in SBF [22]. These glasses were also confirmed to bond to living bone though a calcium phosphate layer in vivo [22].

Ceravital®-type glass-ceramic containing apatite was also found to form apatite on its surface in SBF [16] and was confirmed to bond to living bone through a calcium phosphate layer in vivo [16]. Glass-ceramic A–W forms apatite on its surface in SBF, as shown in Fig. 1 [11] and Fig. 2 [13], and was confirmed to bond to living bone through the apatite layer in vivo, as shown in Fig. 3 [9,23]. In contrast, as described above, glass-ceramic A–W (Al) does not form an apatite layer on its surface in SBF [11], does not have apatite form on its surface in vivo and does not bond to living bone [14]. The apatite forming ability of Bioverite®-type glass-ceramic containing apatite and phlogopite has not been examined in SBF, but it has been confirmed to bond to living bone through a calcium phosphate layer [24].

Sintered hydroxyapatite was also found to have apatite form on its surface in SBF [25,26] and was confirmed to bond to living bone through an apatite layer in vivo [23]. Apatite/β-tricalcium phosphate biphasic ceramic was also found to have an apatite layer form on its surface in SBF [4] and was confirmed to bond to living bone through the apatite layer in vivo [4]. Calcium sulfate was also found to form an apatite on its surface in SBF as well as in vivo [27].

For composites, a composite in which glass-ceramic A–W particles are dispersed in a polyethylene matrix was also found to have apatite form on its surface in SBF [28] and bonded to living bone [29]. For all these materials, apatite formation on their surfaces in SBF is well correlated with their in vivo bone bioactivities.

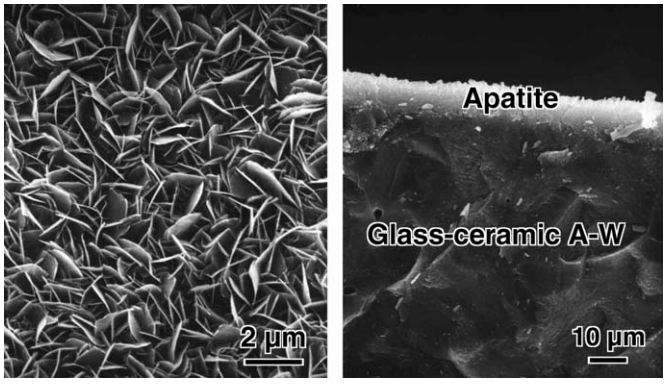

Fig. 1. Scanning electron micrograph of surface (left) and cross section (right) of apatite layer formed on glass-ceramic A–W in SBF [11].

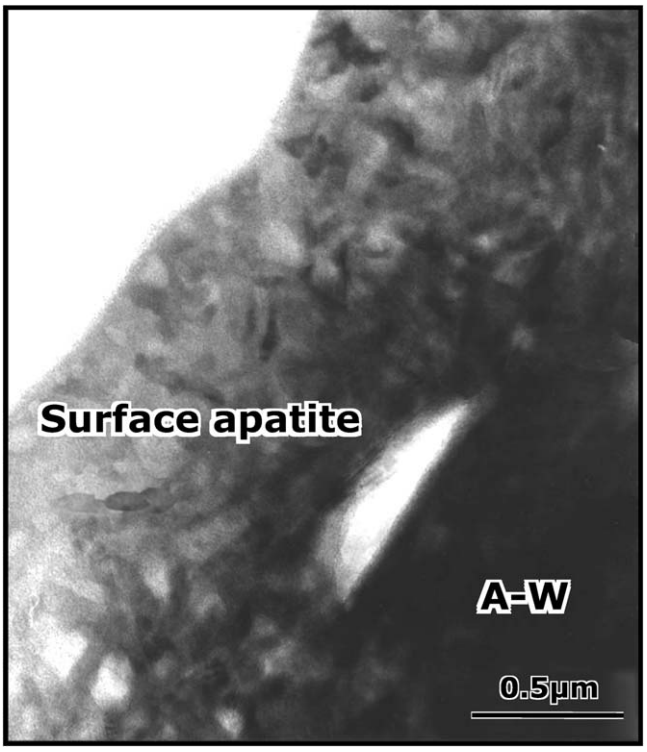

Fig. 2. Transmission electron micrograph of cross section of apatite layer formed on glass-ceramic A–W in SBF [13].

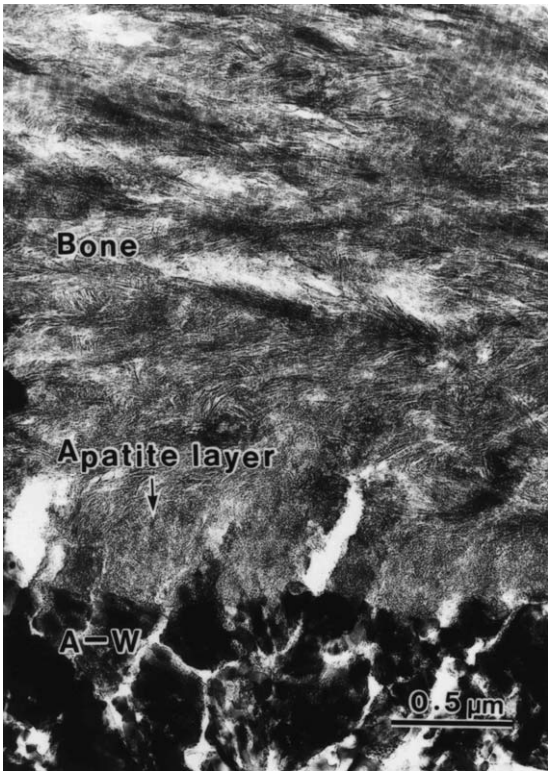

Fig. 3. Transmission electron micrograph of interface between glass-ceramic A–W and rat tibia [23].

However, both β-tricalcium phosphate and natural calcite do not have apatite form on their surfaces in SBF [30,31] or in vivo. [32–34], but despite this, they bond to living bone [32–34]. These results might be related to the high resorbability of these materials. In contrast, natural abalone shell has apatite form on its surface in SBF [31], but does not bond to living bone [35], which might be attributed to antibody reactions to proteins in the shell.

In some reports, SBF with ion concentrations 1.5 times those of SBF (1.5 SBF) has been used when evaluating the in vivo bone bioactivity of a material. There is, however, no correlation between apatite formation on a material in 1.5 SBF with its in vivo bone bioactivity.

It can be said from these results that a material able to have apatite form on its surface in SBF can bond to living bone through the apatite layer formed on its surface in the living body, as long as the material does not contain any substance that induces toxic or antibody reactions.

4. Quantitative correlation of apatite formation in SBF with in vivo bone bioactivity

In 1995, Kim et al. [36] showed that P_2O_5-free Na_2O–CaO–SiO_2 glasses of a wide compositional range have apatite form on their surfaces in SBF, and their apatite forming abilities vary largely with their compositions: i.e. the soaking time in SBF required for apatite formation on their surfaces increased form 0.5 d to longer than 28 d with SiO_2 contents increasing from 50.0 to 70.0 mol% with equal molar concentrations of Na_2O and CaO. Granular particles of these glasses were implanted into holes in rabbit tibiae. The depth of bone growth from the periphery to the interior of the holes at 3 and 6 weeks after implantation increased with the increasing apatite-forming ability of the glasses in SBF at the respective implantation times, as shown in Fig. 4 [37]. The apatite-forming abilities of hydroxyapatite (HA), glass-ceramic A–W and Bioglass in SBF is reported to increase with the order HA < A–W < Bioglass [10,25]. According to Oonishi et al., the depth of bone growth from the periphery to the interior of holes filled with these materials in the tibiae of rabbit also increased in the order HA < A–W < Bioglass [38].

It can be said from these results that the degree of ability for apatite to form on the surface of a material in SBF can predict the degree of in vivo bone bioactivity of the material. A material able to form apatite on its surface in SBF in a short period bonds to living bone in a short period, as a result of apatite formation on its surface in a shorter period within the living body.

5. Development of novel bioactive materials based on apatite formation in SBF

It was shown that CaO and P_2O_5-based glasses in the system CaO–SiO_2–P_2O_5 do not have apatite form on their surfaces in SBF, whereas it forms on CaO and SiO_2-based

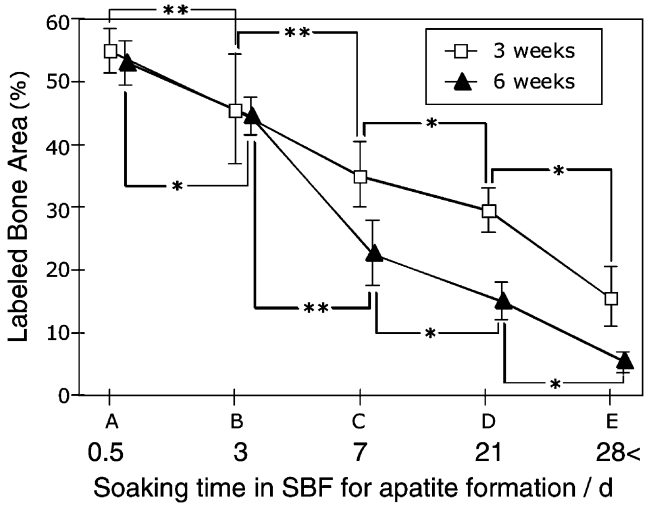

Fig. 4. Dependence of bone formation in a hole of rat tibia filled with glasses on apatite-forming ability of glasses in SBF. Apatite-forming ability increases with decreasing soaking time in SBF for apatite formation [37]. $^{*}p<0.05$, $^{**}p<0.001$.

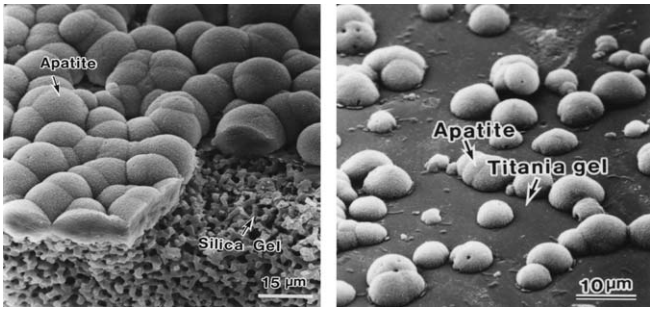

Fig. 5. Scanning electron micrograph of apatite formed on silica gel (left) and titania gel (right) in SBF [46,47].

glasses [39]. The apatite forming ability of a CaO–SiO_2 glass in SBF decreased with the addition of Fe_2O_3 to the glass, and increased with the addition of Na_2O or P_2O_5 [40]. These results then correlated well with in vivo bone bioactivity of the glasses [41,42]. Based on these results, a bioactive ferrimagnetic glass-ceramic containing magnetite in a CaO–SiO_2-based glassy matrix was developed [43,44]. This glass-ceramic can be used as thermoseeds for hyperthermic treatment of cancer [45].

Among the metallic oxide gels prepared using a sol–gel method, those consisting of SiO_2 [46], TiO_2 [47], ZrO_2 [48], Nb_2O_5 [49] and Ta_2O_5 [50] were found to have apatite form on their surfaces in SBF, as shown in Fig. 5, but apatite did not form on gels consisting of Al_2O_3 [47]. These results indicated that Si–OH, Ti–OH, Zr–OH, Nb–OH and Ta–OH groups on the surfaces of these gels are effective for inducing apatite formation on their surfaces in the body environment.

Based on these results, it was speculated that if titanium metal, its alloys and tantalum metal form a sodium titanate or tantalate layer on their surfaces by treatment with a NaOH solution and subsequent heat treatment, they could

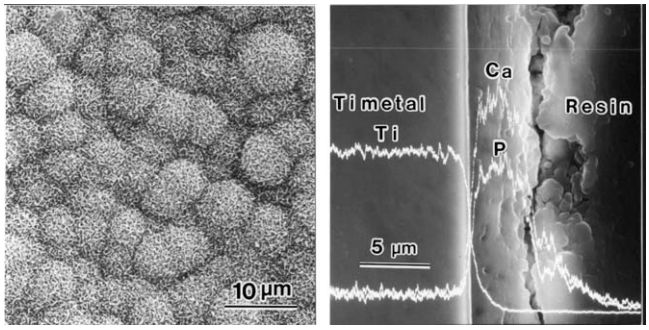

Fig. 6. Scanning electron micrograph of surface (left) and cross section (right) of apatite layer layer formed on NaOH- and heat-treated Ti metal in SBF [51].

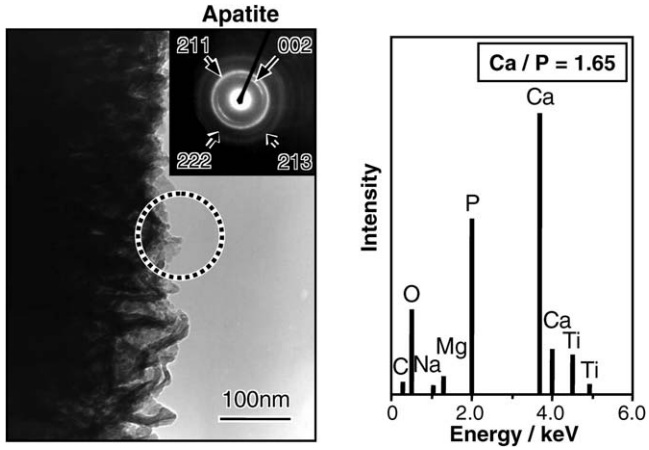

Fig. 7. Transmission electron micrograph (left) and energy dispersive X-ray spectrum (EDX) (right) of apatite formed on NaOH- and heat-treated Ti metals in SBF (dotted circle: area of electron diffraction and EDX analysis) [33].

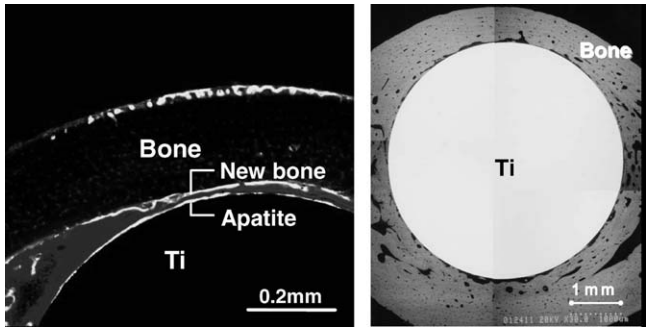

Fig. 8. Confocal laser scanning micrograph (left) and scanning electron micrograph (right) of cross section of NaOH- heat-treated Ti metal rod implanted into rabbit femur for 3 (left) and 12 (right) weeks [54].

form an apatite on their surfaces in SBF, and tightly bond to living bone through the apatite layer formed on their surfaces in the living body. Thus, treated metals had apatite form on their surfaces in SBF [51–53], as shown in Figs. 6 and 7, as well as in vivo [54,55] and, as expected, were tightly bonded to living bone, as shown in Fig. 8 [54]. The bioactive titanium metal thus developed has been applied to artificial hip joints, and a clinical trail of 70 patients has successfully concluded.

6. Conclusion

It is apparent from the results described above that a material able to have apatite form on its surface in SBF has apatite produced on its surface in the living body, and bonds to living bone through this apatite layer. This relationship holds as long as the material does not contain a component that induces toxic or antibody reactions. There are a few materials that directly bond to living bone without the formation of detectable apatite on their surfaces. Despite this limitation, examination of apatite formation on the surface of a material in SBF is useful for predicting the in vivo bone bioactivity of the material, not only qualitatively but also quantitatively. This method can be used for screening bone bioactive materials before animal testing and the number of animals used and the duration of animal experiments can be remarkably reduced by using this method, which can assist in the efficient development of new types of bioactive materials.

Appendix A. Recipe for preparing simulated body fluid (SBF) and procedure of apatite-forming ability test

A.1. Preparation of simulated body fluid (SBF)

A.1.1. Reagents for SBF

The following powder reagent grade chemicals have to be stocked in a desiccator. Ion-exchanged and distilled water is used for the preparation of SBF:

(1) sodium chloride (NaCl),
(2) sodium hydrogen carbonate ($NaHCO_3$),
(3) potassium chloride (KCl),
(4) di-potassium hydrogen phosphate trihydrate ($K_2HPO_4.3H_2O$),
(5) magnesium chloride hexahydrate ($MgCl_2.6H_2O$),
(6) calcium chloride ($CaCl_2$),
(7) sodium sulfate (Na_2SO_4),
(8) Tris-hydroxymethyl aminomethane: ($(HOCH_2)_3CNH_2$) (Tris),
(9) 1M (mol/l) Hydrochloric Acid, 1M-HCl,
(10) pH standard solution, (pH 4, 7 and 9).

A.1.2. Ion concentrations of SBF

The ion concentrations of SBF are shown in Table A1.

A.1.3. Preparation procedure of SBF

Since SBF is supersaturated with respect to apatite, an inappropriate preparation method can lead to the precipitation of apatite in the solution. Always make sure that the preparing solution is kept colorless and transparent and that there is no deposit on the surface of the bottle. If any precipitation occurs, stop preparing SBF, abandon the

solution, restart from washing the apparatus and prepare SBF again.

In order to prepare 1000 ml of SBF, first of all, put 700 ml of ion-exchanged and distilled water with a stirring bar into 1000 ml plastic beaker. Set it in the water bath on the magnetic stirrer and cover it with a watch glass or plastic wrap. Heat the water in the beaker to 36.5 ± 1.5 °C under stirring.

Dissolve only the reagents of 1st to 8th order into the solution at 36.5 ± 1.5 °C one by one in the order given in Table A2, taking care of the indications in the following list. The reagents of 9th (Tris) and 10th order (small amount of HCl) are dissolved in the following process of pH adjustment:

(a) In preparation of SBF, glass containers should be avoided, but a plastic container with smooth surface and without any scratches is recommended, because apatite nucleation can be induced at the surface of a glass container or the edge of scratches. If the container has scratches, replace it by a new one.
(b) Never dissolve several reagents simultaneously. Dissolve a reagent only after the preceding one (if any) is completely dissolved.
(c) Since the reagent $CaCl_2$, which has great effect on precipitation of apatite, takes usually granular form and takes much time to dissolve on granule at a time, completely dissolve one before initiation of dissolution of the next.
(d) Measure the volume of 1M-HCl by cylinder after washing with 1M-HCl.
(e) Measure the hygroscopic reagents such as KCl, $K_2HPO_4 \cdot 3H_2O$, $MgCl_2 \cdot 6H_2O$, $CaCl_2$, Na_2SO_4 in as short a period as possible.

Set the temperature of the solution at 36.5 ± 1.5 °C. If the amount of the solution is smaller than 900 ml, add ion-exchanged and distilled water up to 900 ml in total.

Insert the electrode of the pH meter into the solution. Just before dissolving the Tris, the pH of the solution should be 2.0 ± 1.0.

With the solution temperature between 35 and 38 °C, preferably to 36.5 ± 0.5 °C, dissolve the reagent Tris into the solution little by little taking careful note of the pH change. After adding a small amount of Tris, stop adding it and wait until the reagent already introduced is dissolved completely and the pH has become constant; then add more Tris to raise the pH gradually. When the pH becomes 7.30 ± 0.05, make sure that the temperature of the solution is maintained at 36.5 ± 0.5 °C. With the solution at 36.5 ± 0.5 °C, add more Tris to raise the pH to under 7.45.

Note 1: Do not add a large amount of Tris into the solution at a time, because the radical increase in local pH of the solution can lead to the precipitation of calcium phosphate. If the solution temperature is not within 36.5 ± 0.5 °C, add Tris to raise the pH to 7.30 ± 0.05, stop adding it and wait for the solution temperature to reach 36.5 ± 0.5 °C.

Note 2: The pH shall not increase over 7.45 at 36.5 ± 0.5 °C, taking account of the pH decrease with increasing solution temperature (the pH falls about 0.05/°C at 36.5 ± 1.5 °C).

When the pH has risen to 7.45 ± 0.01, stop dissolving Tris, then drop 1M-HCl by syringe to lower the pH to

Table A1
Nominal ion concentrations of SBF in comparison with those in human blood plasma

Ion	Ion concentrations (mM)	
	Blood plasma	SBF
Na^+	142.0	142.0
K^+	5.0	5.0
Mg^{2+}	1.5	1.5
Ca^{2+}	2.5	2.5
Cl^-	103.0	147.8
HCO_3^-	27.0	4.2
HPO_4^{2-}	1.0	1.0
SO_4^{2-}	0.5	0.5
pH	7.2–7.4	7.40

Table A2
Order, amounts, weighing containers, purities and formula weights of reagents for preparing 1000 ml of SBF

Order	Reagent	Amount	Container	Purity (%)	Formula weight
1	NaCl	8.035 g	Weighing paper	99.5	58.4430
2	$NaHCO_3$	0.355 g	Weighing paper	99.5	84.0068
3	KCl	0.225 g	Weighing bottle	99.5	74.5515
4	$K_2HPO_4 \cdot 3H_2O$	0.231 g	Weighing bottle	99.0	228.2220
5	$MgCl_2 \cdot 6H_2O$	0.311 g	Weighing bottle	98.0	203.3034
6	1.0M-HCl	39 ml	Graduated cylinder	—	—
7	$CaCl_2$	0.292 g	Weighing bottle	95.0	110.9848
8	Na_2SO_4	0.072 g	Weighing bottle	99.0	142.0428
9	Tris	6.118 g	Weighing paper	99.0	121.1356
10	1.0M-HCl	0–5 ml	Syringe	—	—

7.42±0.01, taking care that the pH does not decrease below 7.40. After the pH has fallen to 7.42±0.01, dissolve the remaining Tris little by little until the pH has risen to ⩽7.45. If any Tris remains, add the 1M-HCl and Tris alternately into the solution. Repeat this process until the whole amount of Tris is dissolved keeping the pH within the range of 7.42–7.45. After dissolving the whole amount of Tris, adjust the temperature of the solution to 36.5±0.2 °C. Adjust the pH of the solution by dropping 1M-HCl little by little at a pH of 7.42±0.01 at 36.5±0.2 °C and then finally adjust it to 7.40 exactly at 36.5 °C on condition that the rate of solution temperature increase or decrease is less than 0.1 °C/min.

Remove the electrode of the pH meter form the solution, rinse it with ion-exchanged and distilled water and add the washings into the solution.

Pour the pH-adjusted solution from the beaker into 1000 ml volumetric flask. Rinse the surface of the beaker with ion-exchanged and distilled water and add the washings into the flask several times, fixing the stirring bar with a magnet as if to prevent it from falling into the volumetric flask.

Add the ion-exchanged and distilled water up to the marked line (it is not necessary to adjust exactly, because the volume becomes smaller after cooling), put a lid on the flask and close it with plastic film.

After mixing the solution in the flask, keep it in the water to cool it down to 20 °C.

After the solution temperature has fallen to 20 °C, add the distilled water up to the marked line.

A.1.4. Confirmation of ion concentrations of SBF

Prepared SBF should have the ion concentrations shown in Table A1. In order to confirm the ion concentrations of the SBF, chemical analysis of the SBF is recommended, because SBF is a metastable solution supersaturated with respect to apatite.

Note: It is also recommended that the apatite-forming ability of standard glasses should be examined in the prepared SBF. Chemical compositions of the standard glasses are shown in Table A3. When standard glasses A–C are soaked in SBF, an apatite layer should be detected by thin-film X-ray diffraction and/or scanning electron microscopy after soaking for 12, 24 and 120 h, respectively.

Table A3
The compositions of the standard glasses in the SiO_2–Na_2O–CaO system

Standard glass	Composition (mol%)		
	SiO_2	Na_2O	CaO
A	50	25	25
B	55	22.5	22.5
C	60	20	20

A.1.5. Preservation of SBF

Prepared SBF should be preserved in a plastic bottle with a lid put on tightly and kept at 5−10 °C in a refrigerator. The SBF shall be used within 30 d after preparation.

A.2. Procedure of apatite-forming ability test

A.2.1. Soaking in SBF

For dense materials, measure the specimen dimensions and calculate the surface area with an accuracy of 2 mm^2 for a thin plate.

Calculate the volume of SBF that is used for testing using the following Eq. (1):

$$V_s = S_a/10, \quad (1)$$

where V_s is the volume of SBF (ml) and S_a is the apparent surface area of specimen (mm^2).

For porous materials, the volume of SBF should be greater than the calculated V_s.

Put the calculated volume of SBF into a plastic bottle or beaker. After heating the SBF to 36.5 °C a specimen should be placed in the SBF as shown in Fig. A1. The entire specimen should be submerged in the SBF.

Note: In rare cases, apatite may homogeneously precipitate in the SBF and can be deposited on the surface of a specimen. Therefore, it is recommended that the specimens be placed in the SBF as shown in Fig. A1(a) or Fig. A1(b). In case of placement as shown in Fig. A1 (b), apatite formation should be examined for the lower surface of the specimen.

After soaking at 36.5 °C for different periods within 4 weeks in the SBF, take out the specimen from the SBF and gently wash it with pure water. The specimen should be dried in a desiccator without heating.

Note 1: Bone bonding materials usually form apatite on their surfaces within 4 weeks.

Note 2: A specimen, once taken out of SBF and dried, should not be soaked again.

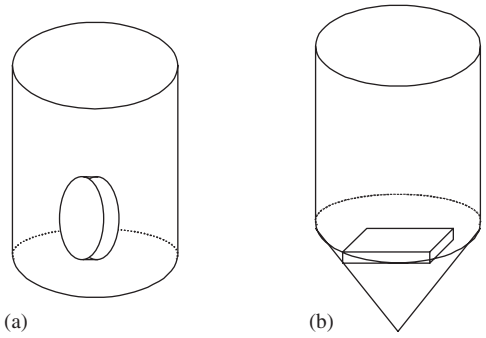

Fig. A1. A specimen in the SBF (Examples).

A.2.2. Surface characterization

Examine the surface of a specimen by TF-XRD and/or scanning electron microscope (SEM) until apatite is detected.

Note 1: The TF-XRD measurement is to be performed in the range of 3–50° in 2 theta (θ) using CuK_α ($\lambda = 0.15405$ nm) radiation as the source at a rate of 2°/min and with 1° glancing angle against the incident beam on the specimen surface.

Note 2: The dried specimen for SEM observation should be thinly metal-coated to induce electro conductivity. The SEM photos should be taken both at high magnifications (around 10,000) and low magnifications (around 1000).

Note 3: The TF-XRD measurement can clearly identify the apatite formation on the specimen. The SEM observation can observe the material formation on the specimen, but can not identify whether the formed material is apatite or not. Therefore, the SEM observation should be accompanied with TF-XRD measurement. However, formed apatite grains and layers have characteristic features to be identified, and the apatite formation is sometimes estimated only on SEM.

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