ROUND-ROBIN TEST OF SBF FOR *IN VITRO* MEASUREMENT OF APATITE-FORMING ABILITY OF SYNTHETIC MATERIALS

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A number of researchers have used a simulated body fluid (SBF) to get an *in vitro* preliminary assessment of bone-bonding ability of an artificial material by examining apatite-forming ability in the SBF, before animal experiments. However, the common method for preparing the SBF and for detecting an apatite formed on a surface of a material in the SBF has not been established yet. The ion concentrations of the conventional SBF are not exactly equal to those of human blood plasma. In the present study, the detailed preparing method, and a newly improved SBF were proposed. *In vitro* assessments of apatite-forming ability in the conventional and proposed SBFs were performed.

(Received June 23, 2004; Accepted July 30, 2004)

INTRODUCTION

It has been revealed that various kinds of materials bond to living bone through a layer of apatite formed on their surfaces in the living body.¹⁻³ It has been also shown that this apatite layer can be reproduced on their surfaces even in an acellular and protein-free simulated body fluid (SBF) with ion concentrations nearly equal to those of human blood plasma, and that apatite thus formed is very similar to the bone mineral in its composition and structure.⁴⁻¹⁴ On the basis of these results, a number of researchers have evaluated *in vivo* bone-bonding ability of a material by examining *in vitro* apatite-forming ability on its surface in the SBF preliminary to animal experiments. It is quite useful to save the lives of test animals, and reduce the experimental period and cost.

It is, however, known that the ion concentrations of conventional SBF (c-SBF) are not exactly equal to those of human blood plasma. Recently, several revised SBFs such as revised SBF (r-SBF) and modified SBF (m-SBF) with ion concentrations equal to and close to those of human blood plasma, respectively, have been proposed.^{15,16} However, the r-SBF and m-SBF with high HCO_3^- concentration tend to deposit calcite in addition to bonelike apatite especially on Ca-based bioactive materials due to the release of Ca^{2+} ion. Therefore, the r-SBF and m-SBF are not the best media for bioactivity assessment.

It is also known that the assessment using SBF does not always show good reproducibility. It usually depends on misunderstanding or the lack of care for

preparing method of the SBF, as its detailed preparing method has not been established yet. Another possible reason lies in the conventional preparing process, i.e., powder dissolving process. It is considered that the radical increase in the local supersaturation with respect to apatite or calcite raised by dissolving powder reagents can lead to the undesirable precipitation of apatite or calcite during the preparation process. Therefore, it is recently proposed that liquid mixing processes is better to reduce the risk of undesirable precipitation.

On the basis of above opinions and previous works, the composition and the preparation procedure were modified and a newly improved SBF (n-SBF) with ion concentrations equal to those of human blood plasma excepting that of HCO_3^- was proposed.¹⁷ The n-SBFs were prepared by two different preparing processes of powder dissolving and liquid mixing processes, respectively. The detailed procedures for preparing the SBFs were also specified. The comparison of *in vitro* measurement of apatite-forming ability was carried out between the c-SBF and n-SBFs.

The result of round robin tests (RRT) of the SBFs for *in vitro* measurements of apatite-forming ability are reported in the present paper. Ten institutes, including universities, companies and public institutes, took part in this RRT, as shown in Table 1. The authors organized and summarized this RRT under the supervision of Prof. Kokubo, who is the inventor of the SBF.⁴ On the basis of the results of the RRT, instructions to prepare stable SBFs with good reproducibility is provided.

Table 1. The type, institutes and principal investigator of RRT

Institutes	Principal investigator
Tokyo Medical and Dental University	K. Yamashita
Tokyo Dental College	M. Yoshinari
Tokyo Institute of Technology	Y. Ohba
Nagoya Institute of Technology	T. Kasuga
Nara Institute of Science and Technology	C. Ohtsuki
Okayama University	A. Osaka
Kobe Steel, Ltd.	J. Suzuki
Mitsubishi Materials Corporation	N. Asaoka
National Institute of Advanced Industrial Science and Technology	Y. Yokogawa
Japan Fine Ceramics Center	M. Mizuno

MATERIALS AND METHODS

The c-SBF and two kinds of proposed n-SBFs were prepared following the detailed instructions. The ion concentrations of them are shown in Table 2. The reagents, purity and quantities for the preparation of 1000 mL of the SBFs are shown in Table 3. All reagents for the preparation of the SBFs were supplied by Nacalai Tesque, Inc., Japan.

The c-SBF is abbreviated from conventional SBF.⁴ It has ion concentrations equal to those of human blood plasma excepting that of HCO_3^- and Cl^- . The concentration of HCO_3^- is lower and Cl^- is higher than that of human blood plasma. It was prepared by dissolving the reagents shown in Table 3 into the solution one by one, i.e., powder dissolving process. It was buffered by tris-hydroxymethyl aminomethane (TRIS) and 1.0-M HCl at pH 7.40 at 36.5 °C. In the process of pH adjustment, TRIS was dissolved little by little into the solution to increase the pH gradually up to 7.45, and then 1M-HCl and TRIS were added alternately, keeping the pH within the range of 7.42 to 7.45, until the whole amount of TRIS was dissolved. Then, pH was adjusted at 7.40 exactly at 36.5 °C finally.

The np-SBF is abbreviated from n-SBF prepared by powder dissolving process in the similar manner to the c-SBF. It is designed to have concentrations of ions equal to those of human blood plasma excepting that of HCO_3^- , the concentration of which is set at the same as that of the c-SBF. It was buffered by

2-(4-(2-Hydroxyethyl)-1-piperazinyl)ethane sulfonic acid (HEPES) and 1.0-M NaOH at pH 7.40 at 36.5 °C. The dissolving order of reagents was modified. The HEPES and NaOH are dissolved first of all to keep the pH constant and NaHCO_3 was dissolved just before the pH adjustment as shown in Table 3 to prevent the volatilization of carbon dioxide (CO_2).

The nl-SBF is abbreviated from n-SBF prepared by liquid mixing process. It has almost same ion concentrations as np-SBF. It was prepared by mixing the Ca and P solutions prepared separately in advance. As shown in Table 2, the Ca solution was designed to have twice higher Ca^{2+} concentration than that of human blood plasma but not to contain HPO_4^{2-} and HCO_3^- to prevent precipitations of calcium phosphate and calcite, while the concentrations of the other ions and pH were designed to be equal to those of human blood plasma. The P solution was designed to have twice higher HPO_4^{2-} and HCO_3^- concentrations than those of human blood plasma but not to contain Ca^{2+} , while the concentrations of the other ions and pH were designed to be equal to those of human blood plasma. This liquid mixing procedure can prevent the local supersaturation with respect to apatite or calcite from increasing radically and reduce the risk of undesirable precipitation during the preparation process.

Ion concentrations of the SBFs were measured by inductively coupled plasma (ICP) emission spectroscopy (IRIS Advantage, Nippon Jarrell-Ash. Co. Japan) and ion chromatograph (DX-100, Dionex Corporation, USA). The pH of SBFs were measured by a pH meter (Mettler-Toledo MA-235, Germany).

Table 2. Nominal ion concentrations of the SBFs in comparison with those of human blood plasma and Ca and P solutions for preparing the nl-SBF

Ion	Concentration/mM				
	Blood Plasma	c-SBF	np-SBF	nl-SBF	
				Ca solution	P solution
Na^+	142.0	142.0	142.0	142.0	142.0
K^+	5.0	5.0	5.0	5.0	5.0
Mg^{2+}	1.5	1.5	1.5	1.5	1.5
Ca^{2+}	2.5	2.5	2.5	5.0	0
Cl^-	103.0	147.8	103.0	103.0	103.0
HCO_3^-	27.0	4.2	4.2	0	8.4
HPO_4^{2-}	1.0	1.0	1.0	0	2.0
SO_4^{2-}	0.5	0.5	0.5	0.5	0.5

Table 3. Reagents, their purities and amounts for preparing 1000 mL of the SBFs

Reagent*	Purity/%	Amount/g			
		c-SBF	Total	Ca solution (500mL)	P solution (500mL)
1.0M-NaOH	---	---	40mL	20mL	20mL
HEPES**	>99.0	---	21.183	10.591	10.591
NaCl	>99.5	8.035	5.404	2.702	2.702
NaHCO ₃	>99.5	0.355	---	---	---
KCl	>99.5	0.225	0.225	0.112	0.112
K ₂ HPO ₄ ·3H ₂ O	>99.0	0.231	0.231	---	0.231
MgCl ₂ ·3H ₂ O	>98.0	0.311	0.311	0.156	0.156
1.0M-HCl	---	39mL	---	---	---
CaCl ₂	>95.0	0.292	0.292	0.292	---
Na ₂ SO ₄	>99.0	0.072	0.072	0.036	0.036
NaHCO ₃	>99.5	---	0.355	---	0.355
TRIS***	>99.0	6.118	---	---	---
1.0M-HCl	---	~0.2mL	---	---	---
1.0M-NaOH	---	---	~4.8mL	~2mL	~3mL

*Listed in sequence of dissolution.

**HEPES: 2-(4-(2-Hydroxyethyl)-1-piperazinyl) ethane sulfonic acid.

***TRIS: Tris-hydroxymethylaminomethane.

Table 4. The compositions of the SiO₂-Na₂O-CaO glasses

Glass	Composition / mol%		
	SiO ₂	Na ₂ O	CaO
Glass A	50	25	25
Glass B	55	22.5	22.5

Two bioactive CaO-SiO₂-Na₂O glasses named glass A and B were used to assess the c-SBF, np-SBF and nl-SBF. It has been reported that these glasses show a correlation between bone-forming ability of materials implanted into a bone defect and apatite-forming ability on its surface in the SBF.¹⁴ Their compositions are shown in Table 4. The glasses with mirror or #400-abraded surface were prepared for each glass. One piece of the glass A was immersed in 20 mL of each SBF at 36.5°C for 6, 12 and 18h and glass B for 12, 18, 24, and 30h, respectively. After removal from each SBF, specimens were washed with distilled water and dried in air. The surfaces of specimens were examined by thin-film X-ray diffraction (TF-XRD: Model-265A, Rigaku Corporation, Osaka, Japan) and scanning electron microscope (FE-SEM: JSM-6330F, JEOL DATUM Co. Ltd., Nagoya, Japan).

RESULTS

It goes without saying that the c-SBF was prepared successfully with taking great care of pH change. The np-SBF and nl-SBF were prepared successfully by the newly proposed procedure as well as c-SBF. However, it took almost twice more time and labor to prepare the nl-SBF than the c-SBF and np-SBF.

Figure 1 shows the changes in pH of the solutions in the preparation process of the c-SBF and np-SBF. The pH became quite low after adding aqueous

1.0M-HCl and the large pH change was involved in the preparing process of the c-SBF. On the other hand, the pH was relatively constant and the small pH change was involved in the preparation process of the np-SBF, because the buffer was added from the beginning. In the preparation process of the nl-SBF, both the Ca and P solutions were prepared showing the quite similar pH changes to the np-SBF. The pH kept 7.40 even after mixing Ca and P solutions to prepare the nl-SBF.

All the as-prepared SBFs were transparent without any precipitation. Figure 2 shows ion concentrations and pH of the SBFs as-prepared and after stored for 4 weeks, in comparison with those of human blood plasma. The as-prepared c-SBF showed ion concentrations almost equal to nominal values listed in Table 2, excepting those of Cl⁻ and HCO₃⁻. The measured Cl⁻ concentration was higher and HCO₃⁻ was lower than the nominal values. On the other hand, the as-prepared np-SBF and nl-SBF showed ion concentrations almost equal to nominal values, including those of Cl⁻ and HCO₃⁻. There was little difference in ion concentrations between the np-SBF and nl-SBF, since the nominal compositions were same. The c-SBF and both n-SBFs kept their ion concentrations and pH unchanged over 4 weeks at 36.5°C. They kept clear and transparent even after the storage for 8 weeks at 36.5°C.

Figure 3 shows the XRD patterns of the surfaces of glass A and B with mirror or #400-abraded surfaces

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after soaked in the c-SBF, np-SBF and nl-SBF for various periods, respectively. Regardless of SBF, the glass A and B showed the first XDR patterns of apatite after soaking of 12h and 18h, respectively. Both of the first detection time of apatite formed on glass A and B was less affected by the difference in the surface finishes.

Figure 1. The pH changes of the solutions in the preparation processes of (a)c-SBF and (b)np-SBF.

Figure 4 shows the SEM images of the surfaces of the glass A and B with mirror and #400-abraded surfaces after soaked in the c-SBF, np-SBF and nl-SBF for 12 and 18 h, respectively. Regardless of SBF, both glass A and B formed apatite uniformly on the whole surfaces of them. However, the apatite formed on the #400-abraded glass surfaces had more cracks and bonded more tightly to the glass specimens than that on the mirror surface glasses.

Figure 5 shows the results of the RRT of *in vitro* assessments of the apatite-forming ability of glass A and B in various SBFs. In total, 9 results of XRD and 11 results of SEM were gathered. The glass A soaked in the c-SBF, np-SBF or nl-SBF showed the quite similar XRD and SEM results, respectively. The glass B soaked in different SBFs also showed the similar results as the glass A. The induction periods to form apatite on the surfaces of the glass A and B, respectively, did not depend on the types of SBFs. While the c-SBF showed the relatively high reproducibility, the np-SBF and nl-SBF showed slightly higher reproducibility than c-SBF, and there were no great differences between the np-SBF and nl-SBF. The SEM analyses tended to detect the apatite formation earlier than XRD. The SEM analyses showed smaller data spread than XRD. The intensity of XRD patterns differed from one institute to another, and some institutes did not showed any XRD patterns. It was found in almost every glass that some portions of apatite layer formed on the glasses were peeled off. The area of the peel-off also differed from one institute to another, and the glass with #400-abraded surface showed smaller area of the peel-off in total than that with mirror surface. It became quite difficult to get SEM images of the glass surfaces with the peel-off, as it induced the intense charge up.

As for the preparation of the nl-SBF, a lot of comments were received that the nl-SBF is not easy-to-prepared, as it took almost twice more time and labor to prepare nl-SBF than the c-SBF and np-SBF.

Figure 2. The ion concentrations and pH of the c-SBF, np-SBF and nl-SBF as-prepared and after stored for 4 weeks, in comparison with those of human blood plasma.

Figure 3. X-ray diffraction patterns of the surfaces of glass A and B with mirror (left) and #400-abraded surface (right) after soaked in c-SBF, np-SBF and nl-SBF for various periods.

		XRD		SEM	
Glass SBFs Time		P*		P*	
Glass A	c-SBF	6h	2/9	5/11	5/11
		12h	7/9	10/11	10/11
		18h	7/9	11/11	11/11
	np-SBF	6h	4/9	7/11	7/11
		12h	9/9	11/11	11/11
		18h	9/9	11/11	11/11
	nl-SBF	6h	3/9	6/11	6/11
		12h	8/9	11/11	11/11
		18h	8/9	11/11	11/11
Glass B	c-SBF	12h	1/9	1/11	1/11
		18h	5/9	8/11	8/11
		24h	8/9	11/11	11/11
		30h	7/9	11/11	11/11
	np-SBF	12h	0/9	0/11	0/11
		18h	8/9	10/11	10/11
		24h	7/9	10/11	10/11
		30h	7/9	10/11	10/11
	nl-SBF	12h	0/9	1/11	1/11
		18h	7/9	9/11	9/11
		24h	7/9	10/11	10/11
		30h	8/9	10/11	10/11

P*: The rate of institutes which detect the apatite by XRD and SEM.
(The number of institutes which observed the apatite formation / Total institutes that took part in the RRT.)

Figure 5. Results of SBF round robin tests

Figure 4. SEM images of the surfaces of the glass A and B with mirror (left) and #400-abraded surfaces (right) after soaked in c-SBF, np-SBF and nl-SBF for 12 and 18 h.

DISCUSSION

Generally, the SBFs such as c-SBF and n-SBFs are metastable solutions supersaturated with respect to apatite. An inappropriate preparation method can lead to the homogeneous nucleation of apatite in the solution. However, the detailed instructions for

preparing the SBFs are not always well known all over the world. Sometimes, unstable SBFs were prepared by lack in care of instructions. Especially in the preparation of the c-SBF, the time to prepare it and the dissolution processes of CaCl_2 and TRIS are quite important. To prepare the c-SBF, the pH is once lowered by the addition of 1.0M-HCl as shown in Figure 1. This can prevent the undesirable precipitation of apatite when the CaCl_2 is added, but lead to the volatilization of CO_2 , simultaneously. Therefore, it is necessary to complete the preparation of the c-SBF without interruptions in a given period every time. In addition, if a large amount of CaCl_2 or TRIS were added into the solution at one time, the local Ca concentration or pH of the solution increased radically to induce undesirable precipitations of calcite or apatite. Therefore, it is necessary to dissolve these reagents into the solution little by little. It is also necessary not to raise the pH over 7.45 by adding TRIS, since the c-SBF becomes highly supersaturated with respect to the apatite. Remaining TRIS and 1.0M-HCl have to be added alternately into the solution keeping the pH within the range of 7.42 to 7.45, until the whole amount of TRIS is dissolved. Then, the pH is finally adjusted to 7.40 exactly at 36.5°C. If the c-SBF is preparing in this manner, stable c-SBF can be prepared.

However, the concentration of HCO_3^- in the c-SBF is lower and Cl^- is higher than nominal concentration. This is because the Cl^- concentration derived from 40 mL of aqueous 1.0M-HCl was not included in the nominal value, and that a portion of HCO_3^- volatilized as CO_2 gas when the pH became quite low after adding 1.0M-HCl in the preparing process of the c-SBF. Therefore, the np-SBF was proposed to bring the Cl^- concentration equal to that of human blood plasma and HCO_3^- concentration close to nominal value by changing the buffer. The HEPES buffer and NaOH are dissolved first of all to keep the pH constant and NaHCO_3 was dissolved just before the pH adjustment to prevent the volatilization of CO_2 . Figure 2 proves that the modified preparing process can prevent its volatilization. Therefore, the proposed np-SBF was prepared successfully.

Additionally, some say that the conventional powder dissolving process is fundamentally at risk of the undesirable precipitations, since the radical increases in local pH or concentrations around the dissolving powders are inevitable. It is difficult to prepare constantly the SBFs with nominal concentrations and good reproducibility of the apatite-forming ability assessment. Therefore, the liquid mixing process was also proposed to prepare the nl-SBF. Figure 2 showed that the proposed nl-SBF was also prepared successfully.

Using the c-SBF and proposed np-SBF and nl-SBF, the apatite-forming ability tests were performed. Since all the glass A and B formed apatite regardless of SBF as shown in Figure 3 and 4, it is reasonable to say that both the np-SBF and nl-SBF can be also used for the apatite-forming ability assessments. The formed apatite tended to bond more tightly to the glass with

#400-abraded surface than that with mirror surface, since the mechanical interlock between the glass and the apatite is created only on the abraded surfaces. Therefore, the #400-abraded surface is better than mirror surface for the assessments with high reproducibility.

Since the RRT clarified that both glass A and B showed the quite similar results regardless of SBFs as shown in Figure 5, it is proved that both the np-SBF and nl-SBF can be also used for the apatite-forming ability assessments. The reproducibility of the c-SBF was improved and was higher than originally expected, since all institutes taking part in the RRT prepared the c-SBF following the detailed instructions, which was briefly written above. It is considered that the appropriate procedure for preparing the c-SBF can control the volatilization of CO_2 to some extent and made the RRT data spread lower. On the other hand, the reproducibility of the np-SBF and nl-SBF was quite similar and almost all the institutes showed the good reproducibility of the n-SBFs in the RRT, since the procedure for preparing the n-SBFs involves little volatilization of CO_2 originally. This indicates the SBFs prepared in an appropriate manner following the instructions can show good reproducibility regardless of the preparing procedure.

The first detection time of apatite formation, however, depends on the analyzing apparatus. The SEM can detect a quite small particle of apatite, while the XRD cannot detect until a certain area are covered. The SEM results less affected by the equipment, while the XRD results largely depend on the experimental conditions and device such as type and power of X-ray lamp. Therefore, the SEM tended to detect the apatite formation earlier and showed smaller data spread than XRD. Considering above reasons, it can be said that the apatite-forming ability does not differ by types of SBFs. It is, however, better to use the n-SBF, if you intend to prepare the apatite, whose composition is close to that of human natural bone in addition to the apatite-forming ability assessments. Consequently, the results of the RRT performed in various institutes concluded that the proposed np-SBF and nl-SBF as well as c-SBF could be used for the apatite-forming ability assessments.

CONCLUSION

The proposed np-SBF and nl-SBF as well as c-SBF with good reproducibility were prepared successfully. The bioactive $\text{CaO-SiO}_2\text{-Na}_2\text{O}$ glasses soaked even in the proposed np-SBF or nl-SBF as well as c-SBF showed quite similar apatite-forming ability. All SBFs such as c-SBF, np-SBF and nl-SBF can be used for *in vitro* apatite-forming ability assessments preliminary to animal experiments.

ACKNOWLEDGEMENT

The authors would like to express great appreciation to Dr. K. Yamashita, Dr. M. Yoshinari, Dr. S. Takemoto, Dr. Y. Ohba, Dr. T. Kasuga, Dr. C. Ohtsuki, Dr. A. Osaka, Dr. J. Suzuki, Dr. N. Asaoka, Dr. Y. Yokogawa and their colleagues for cooperating and participating in the RRT of the SBFs and all of the researchers for providing many opinions about the SBFs.

We also thank to Dr. T. Miyazaki (Kyushu Institute of Technology), Dr. M. Uchida, Dr. A. Oyane (National Institute of Advanced Industrial Science and Technology), Dr. M. Kamitakahara (Nara Institute of Science and Technology), Dr. H.-M. Kim (Yonsei University) and Dr. M. Kawashita (Kyoto University) for cooperating to prepare the detailed instructions for preparing SBFs.

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