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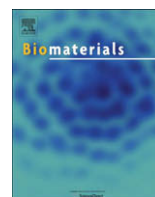
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Leading Opinion

Can bioactivity be tested in vitro with SBF solution? ☆☆☆

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ABSTRACT

A large part of the scientific community has accepted the paradigm that a simulated body solution (SBF) can be used to test the bioactivity of a material. This is exemplified by the rapidly increasing number of publications using this test. The aim of this document is to demonstrate that (i) there is presently not enough scientific data to support this assumption, and (ii) even though the assumption was valid, the way the test is generally conducted leaves room for improvement. Theoretical arguments and facts supporting these statements are provided, together with possible improvements of the proposed bioactivity test.

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1. Introduction

1.1. A short history of SBF

Two decades ago, Kokubo et al. [1,2] used SBF to perform in vitro simulations of in vivo conditions. In 2003 a revised SBF solution was proposed to take into account the fact that a large proportion of calcium and magnesium species present in serum is bound to proteins and hence unavailable for apatite precipitation [3]. The revised SBF solution had a 40% lower calcium concentration and a 33% lower magnesium concentration.

Since 1987, the use of SBF for bioactivity testing has exploded. A search in Scopus (www.scopus.com) using the keywords “bioactivity” and “simulated body fluid” (in all fields) leads to 1975 hits (Jan 5, 2009) with 379 hits in 2008. In 2006, Kokubo and Takadama

reviewed the topic in a paper entitled “How useful is SBF in predicting in vivo bone bioactivity?” [1] and reiterated the statement that SBF could be used to test bioactivity. After 20 years of research in this field, the opinion shared by a large part of the biomaterials community is that the formation of apatite on a material dipped in SBF is a proof of its bioactivity and can be used to anticipate its bone bonding ability in vivo.

1.2. Bioactivity

According to the ESB consensus conference of 1987 [4], a bioactive material is “one which has been designed to induce specific biological activity”. Obviously, there has been a drift of meaning over time, because Kokubo and Takadama [1] consider bioactive materials as bone bonding materials. More specifically, these authors state that “...the essential requirement for a material to bond to living bone is the formation of bone-like 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.” Thus, according to Kokubo and Takadama’s definition of bioactivity, a bioactive material is a material on which bone-like hydroxyapatite will form selectively after it is immersed in a serum-like solution [1,5]. Despite the absence of recommendation of Kokubo regarding the CO₂ partial pressure ($p(\text{CO}_2)$), it is worth mentioning that the use of physiological conditions implies that the test should be performed at $p(\text{CO}_2) = 0.05$ atm (5%) since human serum is in equilibrium with such a partial pressure.

☆ *Editor’s Note:* This paper is one of a newly instituted series of scientific articles that provide evidence-based scientific opinions on topical and important issues in biomaterials science. They have some features of an invited editorial but are based on scientific facts, and some features of a review paper, without attempting to be comprehensive. These papers have been commissioned by the Editor-in-Chief and reviewed for factual, scientific content by referees.

☆☆ This letter is a response to the paper entitled “How useful is SBF in predicting in vivo bone bioactivity” by T. Kokubo and H. Takadama [1].

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2. Crystallization theory

Crystallization theory suggests that bone bonding ability (or bioactivity) and apatite formation in SBF solution are two distinct phenomena. To explain this statement, it is necessary to review basic principles of crystallization theory and relate these principles to the SBF-based bioactivity test.

Thermochemical calculations show that serum and SBF are supersaturated towards apatite crystals [6]. In other words, the system is metastable and will eventually become thermodynamically stable by forming apatite crystals. It is just a matter of time [7,8]. This time, called induction or nucleation time, depends not only on the ease but also on the rate at forming crystal nuclei large enough to be thermodynamically stable and hence large enough to grow (the size above which crystals are stable is generally referred as critical size). So, when apatite crystals form or precipitate on a piece of material dipped in serum or SBF, it simply means that the dipping time was longer than the induction time. Understanding the factors affecting nucleation is therefore essential to understand the outcome of bioactivity experiments performed with SBF.

2.1. Critical size nucleus

In order for a stable crystal to form, it has to overcome the activation energy for crystallization. This energy is the result of the balance between the energy increase due to the formation of a new solid-solution interface and the energy decrease due to the crystal formation. Thus, as the surface to volume ratio is proportional to $1/r$ where r is the particle radius, a minimal (critical) particle radius, r_c , must be reached [8]:

$$r_c = 2\beta_a\sigma V / (3\beta_v RT \ln S)$$

In this equation, β_a and β_v are geometric factors related to the area and the volume of the precipitating nuclei, σ is the free energy per unit area of the nucleus-solution interface, V is the molar volume of the precipitate, T is the temperature and R is the gas constant; S is the saturation ratio, i.e. the ratio of the actual concentration of dissolved precipitate to its concentration at thermodynamic equilibrium. This equation is valid for nuclei forming in a solution, and is referred to as homogeneous nucleation. When nuclei are formed on a solid surface, one refers to heterogeneous nucleation. In that case, the surface free energy between nucleus and solid must also be considered. Since it is generally lower than σ , the critical size is lower and nucleation is easier. In that context, it is interesting to note that Kokubo and Takadama [1] advice to perform bioactivity tests in “a plastic container with smooth surface and without scratches (...) because apatite nucleation can be induced at the surface of a glass container or the edge of scratches”.

2.2. Production rate of critical size nuclei

Beside the ease at nucleating a critical size nucleus, a second aspect of importance for crystallization is the rate at which crystal size nuclei are generated. This rate can be written as [8]

$$J(t) = J_0 \exp(-t/\tau)$$

where J_0 is the steady state nucleation rate, t is the time and τ is the induction time or time constant which is itself expressed as

$$t = 6d^2 n^* / (D \ln S)$$

where d is the molecular diameter, n^* is the critical number of atoms, molecules or ions in the critical size nucleus, D is the diffusion coefficient; S is the saturation ratio already defined above.

3. Apatite formation in SBF and crystallization theories

Relating these theories to dipping tests in SBF, it becomes clear that a “bioactive” compound according to Kokubo’s definition [1,5] is a material that accelerates heterogeneous apatite crystallization in a solution supersaturated towards hydroxyapatite. This can be achieved by several strategies: (i) providing apatite nuclei that remove the need to nucleate apatite crystals, (ii) providing a surface with a low interfacial energy with apatite, or (iii) changing the local supersaturation towards apatite precipitation. The first strategy explains why hydroxyapatite is rapidly covered with new apatite crystals [9,10]. The second strategy gives an explanation for the formation of apatite on β -TCP surface [10] (importantly, β -TCP behaves as an “inert” material in SBF since β -TCP is insoluble in SBF [11,12]). It can also be related to the advice of Kokubo and Takadama [1] to use “plastic containers with smooth surface and without any scratches (...) because apatite nucleation can be induced at the surface of a glass container or the edge of scratches”. The third strategy explains why bioglass and other materials such as calcium sulphate hemihydrate (CSH) are rapidly covered by an apatite layer upon immersion in SBF. Since several sub-strategies exist, the third strategy is discussed in more details hereafter.

3.1. Change of local supersaturation

The addition of a solid into SBF can modify the solution composition and hence the supersaturation provided is at least partly soluble in SBF. A local change of supersaturation can either be positive thus favouring apatite formation or negative hence preventing apatite formation. Taking the case of bioglass, this material is basic and hence provokes a local pH increase of SBF [13–19]. This increase can easily reach two pH units which leads to a 10–100-fold decrease of HA solubility [6], and accordingly, a very large acceleration of apatite nucleation. This effect is reinforced by the release of calcium ions from bioglass [14,16].

Another way to modify locally the solution composition is to use a material soluble in SBF that can release large quantities of calcium and/or phosphate ions. This is the case of CSH (solubility close to 100 mM Ca ions – serum: 1.6 mM) [20], calcium sulphate dihydrate (CSD; solubility close to 10 mM Ca ions) [20] and dicalcium phosphate dihydrate (DCPD) [6] despite the fact that in vivo results have shown that CSH, CSD, and DCPD are resorbed too fast to form a direct bond with bone [21–23].

Beside a pH change or a release of calcium and phosphate ions, a material can also change the local saturation by releasing ions incorporated in poorly soluble apatites. For example, the release of fluoride ions from a material could increase the local saturation of SBF towards fluoroapatite. However, to our knowledge, there are no studies related to such a mechanism.

3.2. Volume, size and kinetics effects

In a previous paragraph, it was described how a change of local SBF composition provoked a change of supersaturation resulting in a deceleration or acceleration of apatite precipitation. Obviously, local dynamics can have an impact on the reaction kinetics. For example, Vallet-Regi et al. [14,17] have shown that the use of a dynamic rather than a static incubation solution postponed apatite formation. Similarly, an increase of the material surface area (e.g. using granules instead of dense forms) leads to faster changes of the local SBF composition and hence to an earlier apatite formation [18,19]. Also, a change of the material/liquid ratio is expected to modify the rate of apatite formation. In fact, changes in the experimental settings could provoke a change in the outcome of the test.

4. Bioactivity testing using SBF

In their “leading opinion paper”, Kokubo and Takadama [1] concluded 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 conclusion is contradicted by the observation done with CSH and DCPD, which both show an apatite layer forming in SBF [10,24] but no direct bone bonding in vivo [21–23,25]. These authors also concluded that “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 statement is contradicted by the fact that β -TCP does not always lead to apatite formation in SBF despite its extensive bonding to bone [26,27]. It would also be contradicted by dipping a non-biocompatible apatite forming substance such as cadmium- or lead-containing apatite.

The collection of results presented here indicates that among the few most significant mineral bone substitutes used in vivo (bioglass, β -TCP, CSH, HA, DCPD), bioactivity testing with SBF may lead not only to false positive but also to false negative results. Therefore, it appears correct to state that “in vitro bioactivity tests in SBF solutions cannot be used to predict the in vivo bone bonding ability of a material”.

4.1. Variability of SBF solutions

Since the first disclosure of the composition of SBF [2,5], several new SBF compositions have been proposed [3,28,29]. However, none of these solutions correspond to the composition of human blood serum. The three main differences between SBF solutions and serum are (i) the absence of proteins, whereas they are known to play an essential role in controlling apatite nucleation (nucleation inhibitors) [10,30]; (ii) the addition of TRIS to buffer SBF solutions, and (iii) the absence of control of the carbonate content of SBF solutions, although carbonates act as pH buffer in serum [31]. Thus, current SBF compositions look somewhat arbitrary and often far from the blood serum they are supposed to simulate. The only requirement apparently retained for a SBF to test the “bioactivity” of a material is that it can precipitate hydroxyapatite at the physiological temperature (37 °C).

The supersaturation of SBF solutions with respect to various calcium phosphates has been calculated recently by Lu and Teng [6]. These authors demonstrated that all SBF solutions proposed so far are supersaturated towards HA and OCP precipitation and undersaturated towards DCPD precipitation. Unfortunately, these authors did not consider the carbonate content of the SBF solution despite the fact that blood serum is in equilibrium with a partial pressure of carbon dioxide (CO_2) close to 0.05 atm [31], which is a key factor in pH buffering of blood.

Therefore, any solution that would meet the following criteria could be proposed as an alternative SBF solution: (i) Simplified composition, incorporating only the main inorganic ions present in blood serum (Na^+ , Ca^{2+} , Cl^- , HCO_3^- , HPO_4^-). (ii) Biomimetic composition, mimicking the main features of blood serum (ionic strength around 140 mM, initial Ca concentration around 2.5 mM and pH = 7.4). (iii) Thermochemical status: the solution is in equilibrium with DCPD in physiological conditions ($T = 37$ °C, partial CO_2 pressure = 0.05 atm and pH = 7.4).

A selection of SBF compositions are compared in Appendix I.

4.2. Procedure to produce SBF

The method proposed by Kokubo and Takadama [1] to prepare SBF solution is tricky, which increases the risk of obtaining non

reproducible results: (i) The SBF preparation procedure performed at 37 °C under stirring increases the risk of premature calcium phosphate precipitation. (ii) The solutions are not filtered at any point, although the presence of insoluble contaminants can have a significant effect on precipitation [7]. (iii) The carbonate content of the solutions is not controlled, although it can affect the solution pH (through exchange with the atmosphere) and change the saturation levels of calcium phosphates and carbonates [32].

Ideally, the preparation of SBF solutions should be easy and reproducible. In order to meet this objective, the starting materials could be distributed into two thermodynamically stable stock solutions that could be prepared and stored for reasonable times prior to be used in bioactivity tests. The stock solutions should be prepared in 5% CO_2 atmosphere, should be ultrafiltered and stored in tight bottles in the dark until use. They could be equilibrated at the testing temperature and carefully mixed just before use. An alternative simplified preparation of SBF solutions is proposed in Appendix II.

5. Conclusion

In this paper, it has been shown that the choice of SBF solution for testing the bone bonding ability of materials is arbitrary. Moreover, the protocol proposed by Kokubo and Takadama [1] for preparing SBF solutions leaves room for improvement, since (i) the procedure is long and tricky, (ii) the solutions are not filtered, and (iii) the carbonate content is not controlled. Furthermore, the use of SBF for bioactivity testing leads to false positive and false negative results. Despite these criticisms, the use of an in vitro protocol for testing the bone bonding potential of a material remains a very attractive concept and should be contemplated very carefully. This opinion is shared by numerous scientists as evidenced by the large number of papers describing the use of one or another SBF. However, in our opinion, the basic principles of the method and its actual use in predicting bone bonding ability need further elaboration.

If we assume that bioactivity can be indeed tested in vitro, the following iterative approach to the problem is suggested: (i) Choose the simplest SBF mimicking the main features of blood serum: pH = 7.4 at 37 °C, under $p(\text{CO}_2) = 0.05$ atm, in equilibrium with DCPD. (ii) Dip reference materials (CSD, β -TCP, HA, Ti, bioglass etc...) in the selected solution and correlate their in vitro behaviour with their in vivo behaviour. The surface state of the tested materials should be well characterized, and identical in both in vivo and in vitro tests. (iii) In case no significant correlation is found between the bioactivity tests and the in vivo results, investigate possible effects of additives (e.g. Mg, K, sulfates, proteins, etc...), until consistent results are obtained.

Appendix I. Comparison of selected SBF compositions

Four possible SBF solutions have been compared from the thermodynamic viewpoint, i.e. their saturation level with respect to selected Ca phosphates and calcite, and their potential for hydroxyapatite precipitation in physiological conditions. The calculations have been performed using the computation algorithm of Verecke et al. [32].

Table I summarizes the rationale and the main features of the SBF selection.

Table II presents the compositions of the selected SBF in terms of inorganic ion concentrations: *c-SBF2* corresponds to the Kokubo's corrected SBF [1]; *c-SBF3* is *c-SBF2* equilibrated at 37 °C with $p(\text{CO}_2) = 0.05$ atm; *SBF-JL1* is *c-SBF3* from which K, Mg and sulphate ions have been removed.

Table I
Specific features of selected simulated body fluids.

Solution	Specific features
<i>c-SBF2</i>	Original Kokubo's <i>c-SBF</i> ; fixed carbonate concentration (closed system evolution)
<i>c-SBF3</i>	Original Kokubo's <i>c-SBF</i> ; equilibrated with $p(\text{CO}_2) = 0.05$ atm
<i>SBF-JL1</i>	<i>c-SBF3</i> made free of K, Mg and sulphate; equilibrated with $p(\text{CO}_2) = 0.05$ atm
<i>SBF-JL2</i>	Solution saturated with DCPD, Ca/P = 1.67; equilibrated with $p(\text{CO}_2) = 0.05$ atm
Notice	Initial pH adjusted at 7.40 with hydrochloric acid

The simplest SBF solution that can be thought of should be in equilibrium with dicalcium phosphate dihydrate (DCPD; $\text{CaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) just like blood serum [33]. It should have a pH value close to 7.4, an initial Ca concentration around 2.5 mM and an ionic strength around 140 mM [34]. Furthermore, it should be in equilibrium with the physiological temperature and partial CO_2 pressure ($T = 37^\circ\text{C}$, $p(\text{CO}_2) = 0.05$ atm). Solution *SBF-JL2* matches these criteria.

Table III summarizes the thermochemical status of the solutions before the occurrence of any precipitation, and after equilibration with respect to HA. The expression “closed system evolution” means that no material exchange occurs between solution and environment; specifically, the carbonate content of the system remains constant. Otherwise, the carbonate content is in equilibrium with $p(\text{CO}_2) = 0.05$ atm throughout the process.

Table III shows that all SBFs are initially oversaturated with respect to calcite and to all the selected Ca phosphates except DCPD. In contrast with the other solutions, *c-SBF2* is initially in equilibrium with $p(\text{CO}_2) = 0.006$ atm. The fact that all the selected SBFs are able to precipitate calcite and Ca phosphates other than HA is interesting, in that it opens the possibility to test the selectivity for a given material or surface treatment to nucleate selectively different precipitates, or to test the promoting effect for HA precipitation of soluble additives such as proteins or oligopeptides.

After equilibration with HA, *c-SBF2* and *SBF-JL2* become undersaturated with respect to calcite, in contrast with solutions *c-SBF3* and *SBF-JL1*. Notice that solution *SBF-JL2*, although its initial HA saturation is close to the other solutions, is able to precipitate 36% more HA.

Another interesting point is the pH evolution upon HA equilibration: the pH remains very close to 7.4, except for *c-SBF2*: in this case, a pH drop down to 6.25 occurs, whereas $p(\text{CO}_2)$ increases up to 0.058. This point stresses the importance of conducting bioactivity tests under constant $p(\text{CO}_2)$; this experimental constraint should not be such of a problem, as incubators used for cell culture are commonly equipped with a $p(\text{CO}_2)$ control and monitoring system.

Appendix II. Preparation procedure for SBF solutions

This appendix presents the recipes for preparing selected SBF solutions. The reagents proposed have the same purity as those

Table II
Compositions of selected simulated body fluids.

Ion	Ion concentrations [mM]			
	<i>c-SBF2</i>	<i>c-SBF3</i>	<i>SBF-JL1</i>	<i>SBF-JL2</i>
Na^+	142.00	142.00	142.00	142.00
K^+	5.00	5.00		
Mg^{2+}	1.50	1.50		
Ca^{2+}	2.50	2.50	2.50	2.31
HCO_3^-	4.20	35.23	34.90	34.88
HPO_4^{2-}	1.00	1.00	1.00	1.39
SO_4^{2-}	0.50	0.50		
Cl^-	147.96	117.62	111.00	109.90

Table III
Thermochemical status of selected simulated body fluids ($T = 37^\circ\text{C}$).

	Saturation levels of selected precipitates			
	<i>c-SBF2</i>	<i>c-SBF3</i>	<i>SBF-JL1</i>	<i>SBF-JL2</i>
<i>Initial state</i>				
$S^\circ(\text{Calcite})$	0.12	0.56	0.57	0.55
$S^\circ(\text{DCPD})$	-0.06	-0.08	-0.05	0.00
$S^\circ(\text{DCP})$	0.16	0.16	0.17	0.23
$S^\circ(\text{OCP})$	0.63	0.61	0.64	0.68
$S^\circ(\beta\text{-TCP})$	0.75	0.73	0.75	0.79
$S^\circ(\text{HA})$	1.42	1.40	1.42	1.45
pH	7.40	7.40	7.40	7.40
$p(\text{CO}_2)$ [atm]	0.59%	5.0%	5.0%	5.0%
<i>After equilibration with HA</i>				
$S^\circ(\text{Calcite})$	-0.75	0.32	0.32	-0.29
$S^\circ(\text{DCPD})$	-1.28	-2.02	-2.02	-1.61
$S^\circ(\text{DCP})$	-1.06	-1.79	-1.80	-1.39
$S^\circ(\text{OCP})$	-0.80	-1.12	-1.12	-0.95
$S^\circ(\beta\text{-TCP})$	-0.77	-0.92	-0.92	-0.84
$S^\circ(\text{HA})$	0	0	0	0
pH	6.25	7.39	7.39	7.38
$p(\text{CO}_2)$ [atm]	5.8%	5.0%	5.0%	5.0%
$\Delta W(\text{HA})$ [mg/L]	163.9	167.4	167.4	227.0

$S^\circ(X)$ is the thermodynamic saturation level.

$S^\circ(X) = \Delta G_{\text{diss}}(X)/RT \ln 10$, where $\Delta G_{\text{diss}}(X)$ is the free enthalpy of dissolution of precipitate X, per mole of released primary solutes (e.g. HA , $\text{Ca}_5\text{OH}(\text{PO}_4)_3 \rightarrow 5\text{Ca}^{2+} + \text{OH}^- + 3\text{PO}_4^{3-}$ releases 9 moles of primary solutes per mole of dissolving HA).

$S^\circ(X) > 0$ indicates that compound X can precipitate; $S^\circ(X) = 0$ means that X is in equilibrium with the solution.

$\Delta W(\text{HA})$ [mg/L], amount of precipitable hydroxyapatite.

c-SBF2: closed system evolution (constant total carbonate content); all other solutions are considered to be in equilibrium with $p(\text{CO}_2) = 0.05$ atm.

presented in Table A2 in reference [1]. Besides the solid reagents, a titrated 1.0 M HCl solution would be used. The amounts of reagents are presented in Table IV: the main difference between *c-SBF2* and the Kokubo's recipe is that TRIS is not used in the preparation.

As successive mixing of solid reagents under stirring at 37°C involves the risk of premature precipitation of HA or other potential precipitates, an alternative way for SBF solutions preparation is suggested. It consists in preparing separately in advance two stock solutions, as shown in Table V: Solution A would incorporate all the solid reagents except CaCl_2 , plus half the prescribed HCl solution; Solution B would incorporate CaCl_2 and the other half of the prescribed HCl. Solutions A and B are supposed to be mixed in a 1:1 volume ratio just at the beginning or the bioactivity test. These solutions can be prepared at room temperature in a volumetric flask (1 L). The order of reagents incorporation would not be critical; however, it seems preferable to dilute first the prescribed volume of HCl solution in about 80% of the final volume of distilled water, and then to add the solid reagents (ending with NaHCO_3 for

Table IV
Preparation of selected simulated body fluids.

Starting materials			Weights of starting materials (g/L)			
Formula	MW [g/mol]	Purity [-]	<i>c-SBF2</i>	<i>c-SBF3</i>	<i>SBF-JL1</i>	<i>SBF-JL2</i>
NaCl	58.44	99.5%	8.035	6.213	6.173	6.129
NaHCO ₃	84.01	99.5%	0.355	2.974	2.947	2.945
KCl	74.55	99.5%	0.225	0.225		
$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	177.99	99.0%			0.180	0.249
$\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$	228.22	99.0%	0.231	0.231		
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	203.31	98.0%	0.311	0.311		
CaCl_2	110.99	95.0%	0.292	0.292	0.292	0.270
Na_2SO_4	142.04	99.0%	0.072	0.072		
Volumes of HCl solution (mL/L)						
HCl	Aq. Sol.	1.00 M	0.160	0.850	0.900	0.934

Table V

Dual-solution preparation of selected simulated body fluids (amounts for 2 L final mixture).

Starting materials			Weights of starting materials (g/L)			
			<i>c-SBF3</i>		<i>SBF-JL2</i>	
Formula	MW [g/mol]	Purity [-]	Sol. A	Sol. B	Sol. A	Sol. B
NaCl	58.44	99.5%	6.213	6.213	6.129	6.129
NaHCO ₃	84.01	99.5%	5.948		5.890	
KCl	74.55	99.5%	0.450			
Na ₂ HPO ₄ · 2H ₂ O	177.99	99.0%			0.498	
K ₂ HPO ₄ · 3H ₂ O	228.22	99.0%	0.462			
MgCl ₂ · 6H ₂ O	203.31	98.0%	0.622			
CaCl ₂	110.99	95.0%		0.584		0.540
Na ₂ SO ₄	142.04	99.0%	0.144			
			Volumes of HCl solution (mL/L)			
HCl 1.00 M	Aq. Sol.	[mL/L]	0.850	0.850	0.934	0.934

Solution A). The final volume should be complemented with distilled water after complete dissolution of the solid reagents.

Both solutions A and B are thermodynamically stable, provided they are stored at room temperature in tight sterilizable bottles (preferably in polypropylene). Special care should be taken to prevent biological proliferation in Solution A (e.g. ultrafiltration before storage, storage in a dark place...); adding a minute quantity of preservative (formaldehyde or sodium azide) might also help to keep Solution A sterile.

The following procedure is suggested for the start of the bioactivity test: (1) Equal volumes of solutions A and B are stored separately in tightly closed plastic syringes, and conditioned at 37 °C in the incubator used for the bioactivity test. (2) Prepare a mixing device, consisting in two plastic inlet tubes fixed to a Y connector, the outlet of which is connected to a static mixer (e.g. a plastic tubing of 5 mm inner diameter, filled with a 10-stage Kenics-type "butterfly" mixer). (3) Prepare a vessel dedicated to the bioactivity test (e.g. a standard cell culture flask, preferably new and sterile), containing a specimen of the material to be tested. (4) At the start of the bioactivity test, unplug the two syringes, and connect them to the mixing device through an on-line filter (e.g. 0.25 micron). (5) Eject simultaneously at constant rate the contents of the two syringes through the mixing device into the test vessel, and place it in the incubator. (6) The proposed protocol allows the successive preparation of several test vessels with the same batches of solutions A and B. Thus, the reproducibility of the test should be improved significantly.

References

- [1] Kokubo T, Takadama H. How useful is SBF in predicting in vivo bone bioactivity? *Biomaterials* 2006;27:2907–15.
- [2] Kokubo T, Ito S, Shigematsu M, Sakka S, Yamamuro T. Fatigue and life-time of bioactive glass-ceramic A-W containing apatite and wollastonite. *J Mater Sci* 1987;22:4067–70.
- [3] Oyane A, Kim HM, Furuya T, Kokubo T, Miyazaki T, Nakamura T. Preparation and assessment of revised simulated body fluids. *J Biomed Mater Res A* 2003;65:188–95.
- [4] Williams DF, editor. *Definitions in biomaterials*. Amsterdam–Oxford–New York–Tokyo: Elsevier; 1987.
- [5] Kokubo T. *Bioactive glass ceramics: properties and applications*. *Biomaterials* 1991;12:155–63.
- [6] Lu X, Leng Y. Theoretical analysis of calcium phosphate precipitation in simulated body fluid. *Biomaterials* 2005;26:1097–108.
- [7] Walton AG. *The formation and properties of precipitates*. Huntington, New York: Robert E. Krieger Publishing Company; 1979.
- [8] Ring TA. *Fundamentals of ceramic powder processing and synthesis*. San Diego: Academic Press Limited; 1995.
- [9] Kim HM, Himeno T, Kawashita M, Kokubo T, Nakamura T. The mechanism of biomineralization of bone-like apatite on synthetic hydroxyapatite: an in vitro assessment. *J Royal Soc Interface* 2004;1:17–22.
- [10] Juhász JA, Best SM, Auffret AD, Bonfield W. Biological control of apatite growth in simulated body fluid and human blood serum. *J Mater Sci Mater Med* 2008;19:1823–9.
- [11] Driessens FCM, Verbeeck RM, editors. *Relation between physico-chemical solubility and biodegradability of calcium phosphates*. Amsterdam: Elsevier Science Publishers; 1988.
- [12] Driessens FC, Verbeeck RM, van Dijk JW, Borggreven JM. Degree of saturation of blood plasma in vertebrates with octacalcium phosphate. *Z Naturforsch [C]* 1988;43(1–2):74–6.
- [13] Zhang D, Hupa M, Hupa L. In situ pH within particle beds of bioactive glasses. *Acta Biomater* 2008;4:1498–505.
- [14] Ramila A, Vallet-Regi M. Static and dynamic in vitro study of a sol–gel glass bioactivity. *Biomaterials* 2001;22:2301–6.
- [15] Karlsson KH, Backman R, Hupa M. An equilibrium study of phosphate precipitation on bioactive glass. *Key Eng Mater* 2002;218–220:103–8.
- [16] Jones JR, Sepulveda P, Hench LL. Dose-dependent behavior of bioactive glass dissolution. *J Biomed Mater Res* 2001;58:720–6.
- [17] Izquierdo-Barba I, Salinas AJ, Vallet-Regi IM. Effect of the continuous solution exchange on the in vitro reactivity of a CaO–SiO₂ sol–gel glass. *J Biomed Mater Res* 2000;51:191–9.
- [18] Cerruti MG, Greenspan D, Powers K. An analytical model for the dissolution of different particle size samples of bioglass in TRIS-buffered solution. *Biomaterials* 2005;26:4903–11.
- [19] Cerruti M, Greenspan D, Powers K. Effect of pH and ionic strength on the reactivity of bioglass 45S5. *Biomaterials* 2005;26:1665–74.
- [20] Lindsay WL. *Chemical equilibria in soils*. Wiley; 1979.
- [21] Walsh WR, Morberg P, Yu Y, Yang JL, Haggard W, Sheath PC, et al. Response of a calcium sulfate bone graft substitute in a confined cancellous defect. *Clin Orthop* 2003;406:228–36.
- [22] Theiss F, Apelt D, Brand B, Kutter A, Zlinszky K, Bohner M, et al. Biocompatibility and resorption of a brushite calcium phosphate cement. *Biomaterials* 2005;26:4383–94.
- [23] Apelt D, Theiss F, El-Warrak AO, Zlinszky K, Bettschart-Wolfisberger R, Bohner M, et al. In vivo behavior of three different injectable hydraulic calcium phosphate cements. *Biomaterials* 2004;25:1439–51.
- [24] Chan H, Mijares D, Ricci JL. In vitro dissolution of calcium sulfate: evidence of bioactivity. In: *Transactions - seventh world biomaterials congress, 2004*. Sydney; 2004. p. 627.
- [25] Ohura K, Bohner M, Hardouin P, Lemaître J, Pasquier G, Flautre B. Resorption of, and bone formation from, new beta-tricalcium phosphate-monocalcium phosphate cements: an in vivo study. *J Biomed Mater Res* 1996;30:193–200.
- [26] Ohtsuki C, Kokubo T, Neo M, Kotani S, Yamamuro T, Nakamura T, et al. Bone-bonding mechanism of sintered β -CaO · P₂O₅. *Phosphor Res Bull* 1991;1:191–6.
- [27] Kotani S, Fujita Y, Kitsugi T, Nakamura T, Yamamuro T, Ohtsuki C, et al. Bone bonding mechanism of beta-tricalcium phosphate. *J Biomed Mater Res* 1991;25:1303–15.
- [28] Tas AC. Synthesis of biomimetic Ca-hydroxyapatite powders at 37 degrees C in synthetic body fluids. *Biomaterials* 2000;21:1429–38.
- [29] Kim HM, Miyazaki T, Kokubo T, Nakamura T. Revised simulated body fluid. *Key Eng Mater* 2001;192–195:47–50.
- [30] Garnett J, Dieppe P. The effects of serum and human albumin on calcium hydroxyapatite crystal growth. *Biochem J* 1990;266:863–8.
- [31] Teitelbaum SL. Bone resorption by osteoclasts. *Science* 2000;289:1504–8.
- [32] Vereecke G, Lemaître J. Calculation of the solubility diagrams in the system Ca(OH)₂–H₃PO₄–KOH–HNO₃–CO₂–H₂O. *J Cryst Growth* 1990;104:820–32.
- [33] Eidelman N, Chow LC, Brown WE. Calcium phosphate saturation levels in ultrafiltered serum. *Calcif Tissue Int* 1987;40:71–8.
- [34] Turitto V, Slack SM. Blood and related fluids. In: Black J, Hastings G, editors. *Handbook of biomaterial properties*; 1998. p. 114–24 [Chapter C1].