INTERNATIONAL STANDARD

ISO 23317

First edition
2007-06-15

Implants for surgery — In vitro evaluation for apatite-forming ability of implant materials

Implants chirurgicaux — Évaluation in vitro de la capacité de formation d'apatite des matériaux d'implants

Reference number
ISO 23317:2007(E)

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Published in Switzerland

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Foreword

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ISO 23317 was prepared by Technical Committee ISO/TC 150, *Implants for surgery*, Subcommittee SC 1, *Materials*.

Introduction

It has been revealed that materials of various kinds bind to living bone through a layer of apatite. It has been shown that this apatite layer can be reproduced on their surfaces 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.

This evaluation of apatite-forming ability on implant material in SBF is useful for evaluating its *in vivo* bone-bonding ability preliminary to animal experiments. When a bioactive material is implanted in a living body, a thin layer rich in Ca and P forms on its surface. The material then connects to the living tissue through this apatite layer without a distinct boundary. It has been shown that this apatite layer can be reproduced on the surfaces of materials in SBF as well, and that apatite thus formed is very similar to bone mineral in its composition and structure. As bioactivity increases, apatite forms on the material surface in a shorter time in proportion to this increase. The formation of apatite layers can be detected by thin film X-ray diffraction spectrometry and/or scanning electron microscopy.

NOTE 1 The material which forms apatite on its surface *in vivo* can bond to living bone, since this apatite is biologically active. Their *in vivo* apatite deposition can be reproduced on their surfaces even *in vitro* in SBF. For example, *in vivo* calcification on surfaces of Bioglass®, $CaO\text{-}SiO_2$ glasses, $Na_2O\text{-}CaO\text{-}SiO_2$ glasses, Cerabone®A-W, Ceravital®-type glass-ceramic, sintered hydroxyapatite and alkali-heat-treated titanium metal, are correlated with *in vitro* calcification in SBF. However, this does not exclude the possibility that materials, which do not form apatite on their surfaces *in vivo*, bond to living bone. For example, it is reported that such resorbable materials as beta-tricalcium phosphate ($Ca_3(PO_4)_2$) and calcium carbonate bond to living bone without forming an apatite layer on their surfaces.

NOTE 2 It has been reported that glasses with different compositions in the system $Na_2O\text{-}CaO\text{-}SiO_2$ show a correlation between bone-forming ability of materials implanted into a bone defect of a rabbit and apatite-forming ability on its surface in SBF.

Implants for surgery — In vitro evaluation for apatite-forming ability of implant materials

1 Scope

This International Standard describes the method for detecting apatite formed on a surface of a material in simulated body fluid (SBF).

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods*

ISO 14630:2005, *Non-active surgical implants — General requirements*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 14630 and the following apply.

3.1
apatite
group of calcium-phosphates including bone mineral and the main inorganic constituent of bones and teeth similar to hydroxyapatite, which has the composition $Ca_{10}(PO_4)_6(OH)_2$

NOTE Bone mineral also contains ions such as CO_3^{2-}, F^-, Na^+ and Mg^{2+}.

3.2
apatite-forming ability
capability to develop apatite on the surface

3.3
bioactivity
property that elicits a specific biological response at the interface of the material, which results in the formation of a bond between tissue and material

3.4
induction period
time to detect apatite formation on a surface of a specimen after soaking the specimen in simulated body fluid

3.5
simulated body fluid
SBF
inorganic solution having a similar composition to human plasma without organic components

3.6
standard glass for evaluating apatite-forming ability
class of standard glasses with certain chemical compositions showing given apatite-forming abilities in SBF and when implanted in an animal body

3.7
thin film X-ray diffraction spectrometry
TF-XRD
method for detecting minerals in a thin layer at the surface of a material from a diffraction pattern obtained by X-ray with small glancing angle against the surface of the sample

4 Apparatus

4.1 **Electric balance**, capable of measuring a mass with an accuracy of ± 1 mg.

4.2 **Water bath equipped with magnetic stirrer**, to maintain temperature of the solution within the range 36,0 °C to 40,0 °C.

4.3 **pH meter**, capable of measuring the pH of a solution with an accuracy of ± 0,01.

4.4 **Thin film X-ray diffraction spectrometer**, capable of detecting apatite formed in a thin layer at the surface of a material.

4.5 **Scanning electron microscope**, capable of observing apatite grains and/or layers formed on a plain surface of a material with a magnification up to × 10 000.

5 Test specimen

5.1 Specimen configuration and size

This International Standard allows specimens of any configuration and size derived from implant parts and devices to be used. However, a disc or rectangular plate specimen is highly recommended, because bioactivity of a material is evaluated by confirmation of apatite formed on the surface of the material using TF-XRD and/or SEM. Recommended specimen dimensions are shown in Figure 1.

Dimensions in millimetres

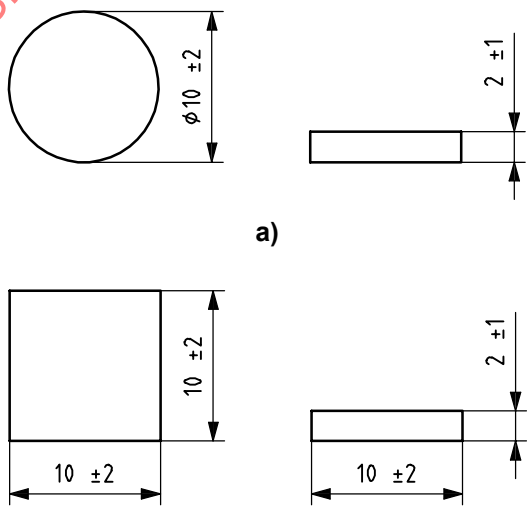


Figure 1 — Recommended specimen dimensions for (a) disc specimen and (b) rectangular specimen

5.2 Specimen preparation

5.2.1 General

This International Standard allows several options for specimen preparation. The specimens should be machined, if necessary, to alter the configurations of original implants.

5.2.2 Basic machining procedure

In the case of a rectangular thin plate specimen as shown in Figure 1 b), the following procedure shall be used. Specimens shall be ground using a diamond wheel of grit size between 120 and 400. Conditions such as depth of cut per pass, wheel speed and others depend on the ground material. Water soluble materials, such as bioactive standard glasses, shall be machined under non-aqueous conditions.

Where a customary machining procedure has been developed that is completely satisfactory for apatite-forming ability testing, this customary procedure can be used.

6 Simulated body fluid

6.1 General

Simulated body fluid (SBF) as defined in Table 1 shall be used.

NOTE 1 For SBF as defined in Table 1, a correlation was observed between *in vivo* bone ingrowth and *in vitro* apatite-forming ability.

NOTE 2 Other kinds of SBFs have been proposed in the literature.

6.2 Reagents for SBF

The following powder reagent grade chemicals shall be stored in a desiccator. Water in accordance with ISO 3696:1987, grade 2, shall be used for the preparation of SBFs.

a) sodium chloride (NaCl)

b) sodium hydrogen carbonate ($NaHCO_3$)

c) potassium chloride (KCl)

d) di-potassium hydrogen phosphate trihydrate ($K_2HPO_4{\cdot}3H_2O$)

e) magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$)

f) calcium chloride ($CaCl_2$)

g) sodium sulfate (Na_2SO_4)

h) tris-hydroxymethyl aminomethane (TRIS): ($(HOCH_2)_3CNH_2$)

i) hydrochloric acid solution, c(HCl) = 1 mol/l.

j) pH standard solutions, (pH 4, 7 and 9)

6.3 Ion concentrations and pH of SBF

The ion concentrations and pH of SBF are shown in Table 1.

Table 1 — Ion concentrations of SBF and human blood plasma

Ion	Concentration (10^{-3} mol) in	
	SBF (pH 7,40)	Blood plasma (pH 7,2 to 7,4)
Na^+	142,0	142,0
K^+	5,0	5,0
Mg^{2+}	1,5	1,5
Ca^{2+}	2,5	2,5
Cl^-	147,8	103,0
HCO_3^-	4,2	27,0
HPO_4^{2-}	1,0	1,0
SO_4^{2-}	0,5	0,5

6.4 Preparation of SBF

6.4.1 General

Since SBF is supersaturated with respect to apatite, an inappropriate preparation method can lead to the homogeneous precipitation of apatite in the solution.

During its preparation the solution shall remain colourless, transparent and without deposit on the surface of the bottle. If any precipitation occurs, stop preparing SBF, abandon the solution and restart by washing the apparatus.

In Table 2, the reagents for the preparation of 1 l of SBF are given in the required order of dissolution.

Table 2 — Order, amount, weighing container, purity and formula weights of reagents for preparing 1 l of SBF

Order	Reagent	Amount g	Container	Purity	Formula weight u
1	NaCl	8,035	weighing paper	99,5 %	58,443 0
2	$NaHCO_3$	0,355	weighing paper	99,5 %	84,006 8
3	KCl	0,225	weighing bottle	99,5 %	74,551 5
4	$K_2HPO_4{\cdot}3H_2O$	0,231	weighing bottle	99,0 %	228,222 0
5	$MgCl_2{\cdot}6H_2O$	0,311	weighing bottle	98,0 %	203,303 4
6	c(HCl) = 1 mol/l	39	graduated cylinder	—	—
7	$CaCl_2$	0,292	weighing bottle	95,0 %	110,984 8
8	Na_2SO_4	0,072	weighing bottle	99,0 %	142,042 8
9	TRIS	118	weighing paper	99,0 %	121,135 6
10	c(HCl) = 1 mol/l	0 to 5	syringe	—	—

6.4.2 Step 1

Put 700 ml of ion-exchanged and distilled water, with a stirring bar, into a 1 l plastic beaker. Set it in the water bath (4.2) on the magnetic stirrer and cover it with a watch glass or plastic wrap. Heat the water in the beaker to 36,5 °C ± 1,5 °C whilst stirring. Annex A shows an example of apparatus for preparing SBF.

6.4.3 Step 2

Dissolve the reagents in the solution at 36,5 °C ± 1,5 °C in the order given in Table 2, whilst considering the following.

a) Glass containers should be avoided. A plastic container, with a smooth surface and without any scratches, is recommended, because apatite nucleation can be induced at the surface of a glass container or the edges of scratches.

b) Dissolve a reagent only after the preceding one (if any) is completely dissolved.

c) The reagent $CaCl_2$ is usually available in granular form and has great effect on the precipitation of apatite. Dissolve the $CaCl_2$ granule by granule.

d) Rinse the graduated cylinder with 1M-HCl before measuring the volume of 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 as quickly as possible.

6.4.4 Step 3

Insert the electrode of the pH meter (4.3) into the solution. Just before dissolving the TRIS, the pH of the solution should be 2,0 ± 1,0.

6.4.5 Step 4

Set the temperature of the solution at (36,5 ± 1,5) °C. If the amount of the solution is smaller than 900 ml, add distilled water up to 0,9 l in total.

6.4.6 Step 5

With the solution temperature between 35 °C and 38 °C, preferably 36,5 ± 0,5 °C, dissolve TRIS into the solution little by little, taking careful note of the pH change. After adding a small amount of TRIS, wait until the reagent is dissolved completely and the pH has become constant. Then add another small amount of TRIS.

It is recommended not to add a large amount of TRIS into the solution all at once, because the radical increase in local pH of the solution could lead to the precipitation of apatite. The following procedure is recommended: If the solution temperature is not within (36,5 ± 0,5) °C, add TRIS to raise the pH to 7,3 ± 0,05, then stop adding and wait for the solution temperature to reach (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. The pH should not increase to 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 at (36,5 ± 1,5) °C].

6.4.7 Step 6

Make sure that the temperature of the solution is maintained at (36,5 ± 0,5) °C. When the pH has risen to 7,45 ± 0,01, stop dissolving TRIS, then add the hydrochloric acid solution, preferably using a syringe to lower the pH to 7,42 ± 0,01, taking care that the pH does not decrease to 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 to the solution. Repeat this process until the whole amount of TRIS is dissolved keeping the pH within the range of 7,42 to 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 adding the hydrochloric acid solution 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.

6.4.8 Step 7

Remove the electrode of the pH meter from the solution, rinse it with distilled water and add the washings to the solution.

6.4.9 Step 8

Pour the pH-adjusted solution from the beaker into a 1 l volumetric. Fix flask. Rinse the surface of the beaker with distilled water several times and add the washings to the flask. Fix the stirring bar with a magnet to prevent it from falling into the volumetric flask.

6.4.10 Step 9

Add the 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 using a plastic film.

6.4.11 Step 10

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

6.4.12 Step 11

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

6.5 Confirmation of ion concentration of SBF

Prepared SBF shall have the ion concentrations given in Table 1. 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.

It is also recommended that the apatite-forming ability of standard glasses in the prepared SBF be examined. Chemical compositions of the standard glasses for evaluating apatite-forming ability are shown in Figure B.1. When standard glasses A, B and C for evaluating apatite-forming ability are soaked in SBF, an apatite layer should be detected by TF-XRD pattern after soaking for about 12 h, 24 h and 120 h.

6.6 Preservation of SBF

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

7 Procedure

7.1 For dense materials, measure the specimen dimensions to an accuracy of ± 0,1 mm and calculate the surface area to an accuracy of 2 mm^2 for a thin plate.

a) **b)**

Figure 2 — Specimens in SBF: (a) disc specimen and (b) rectangular specimen (examples)

7.2 Calculate the volume of SBF that is used for testing using the following equation:

$$v_s = S_a/10$$

where

v_s is the volume of the SBF in cubic millimetres;

S_a is the apparent surface area of the specimen in square millimetres.

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

7.3 Put the calculated volume of SBF into a plastic bottle or beaker. After heating the SBF to 36,5 °C a specimen shall be placed in the SBF as shown in Figure 2. The entire specimen shall be submerged in the SBF.

In rare cases, apatite can 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 Figure 2 a) or Figure 2 b). In case of placement as shown in Figure 2 b), apatite formation should be examined on the lower surface of the specimen.

7.4 After soaking in the SBF at 36,5 °C for different periods within 4 weeks, take out the specimen from the SBF and gently rinse it with pure water.

A soaking time of 4 weeks is recommended.

The specimen shall then be dried in a desiccator without heating. A specimen, once taken out of SBF and dried, shall not be soaked again.

NOTE Bone bonding materials usually form apatite on their surfaces within 4 weeks.

7.5 Examine the surface of a specimen by thin film X-ray diffraction (TF-XRD) and/or scanning with an electron microscope (4.5) until apatite is detected.

It is recommended to perform the TF-XRD measurement in the range of 3 °C to 50 °C in 2 theta (θ) using CuKα (λ = 0,154 05 nm) radiation as the source at a rate of 2°/min and a 1° glancing angle against the incident beam on the specimen surface.

The dried specimen for scanning electron microscope (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 × 1 000).

NOTE 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 cannot identify whether apatite is formed or not. Therefore the SEM observation should be accompanied with the 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.

8 Test report

The test report shall include the following information:

a) all relevant material data including vintage, billet or component;

b) specimen configurations and dimensions;

c) specimen preparation procedure, including machining conditions for specimen surfaces;

d) porosity of material as a percentage (optional);

e) volume of SBF used in the measurement;

f) soaking temperature of SBF (°C);

g) method of detecting the apatite on a specimen surface (TF-XRD and/or SEM);

h) measurement conditions for TF-XRD with diffraction patterns and/or observation conditions for SEM with microphotographs showing the existence of apatite;

i) presence or absence of apatite at each period; or estimated induction period (optional);

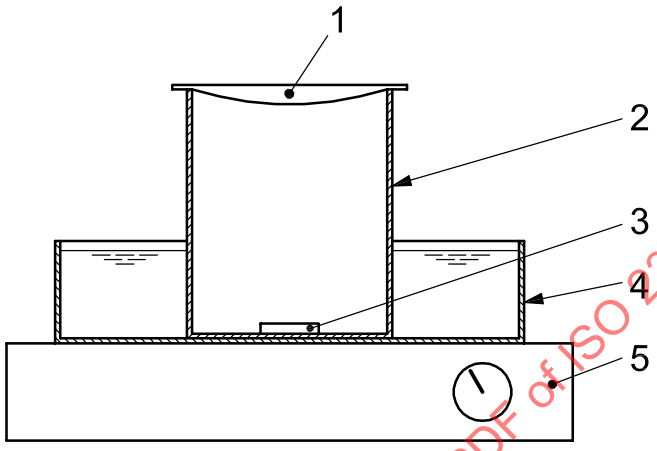
j) number of specimens per condition;

k) name of laboratory and date of the test;

l) reference to this International Standard, i.e., ISO 23317:2007.

Annex A
(informative)

Apparatus for preparing SBF



Key

1 watch glass
2 polyethylene beaker
3 magnetic bar
4 water bath
5 magnetic stirrer

Figure A.1 — Example of apparatus for preparing SBF

Annex B
(informative)

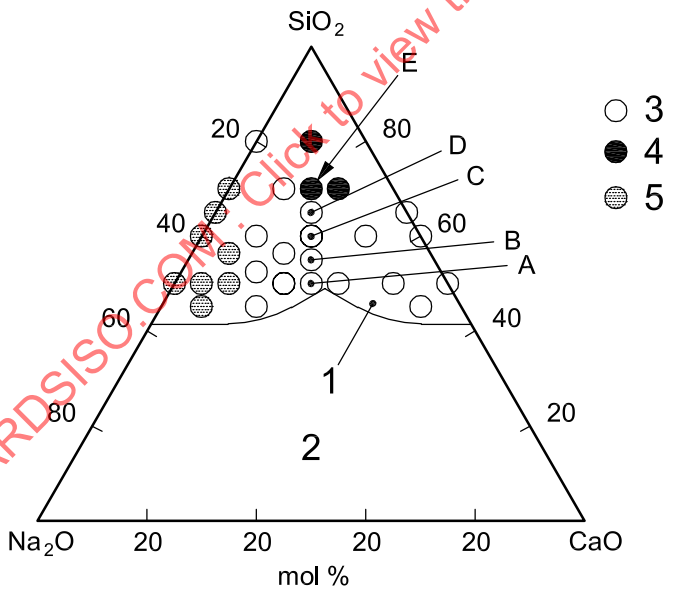
Preparation of standard glasses for evaluating apatite-forming ability

The compositions of the standard glasses are given in Table B.1. The fine grade reagents of SiO_2, Na_2CO_3 and $CaCO_3$ should be used to prepare standard glasses. See Figure B.1.

Prepare the reagents in the amounts listed in Table B.2, taking into consideration the mass loss after burning at selected temperatures. (Alternatively, it is desirable to use the pre-heated reagents in order to eliminate a mass loss; e.g., Na_2CO_3, $CaCO_3$ and SiO_2 are pre-heated at 1 000 °C for 10 h, 600 °C for 2 h and 550 °C for 1 h, respectively.)

After being measured, reagents are gathered in an alumina mortar and mechanically well mixed by use of a pestle for 30 min.

Powders (for glasses A, B and C) in a platinum crucible are melted at 1 400 °C for 1,5 h and then poured out on to a stainless steel plate with spacers 2 mm thick set on the plate. The glass is pressed immediately with another stainless steel plate and formed into a plate 2 mm thick. The glass plates are then moved to another stainless steel plate pre-heated to 500 °C. After annealing, the glasses are cut into specimens under a non-aqueous cooling medium, polished with No. 400 abrasive paper and finally washed in acetone in an ultrasonic bath.



Key

1 glass formation
2 no glass formation
3 apatite formation
4 no apatite formation
5 dissolution

Figure B.1 — Standard glasses in the Na_2O-CaO-SiO_2 system and apatite-forming ability after soaking in simulated body fluid