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Assessment of *in vitro* testing approaches for bioactive inorganic materials

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ABSTRACT

This paper deals with non-standard *in vitro* testing of bioactive inorganic materials shaped as granules or scaffolds. The ISO 23317 standard describes the *in vitro* test arrangement of bulk bioactive materials under static conditions. However, this norm has not dealt with bioactive materials shaped as granules (with large surface area) that are commonly used in clinical practice. We found that in the case of highly reactive (bioactive) materials, the biogenic elements were exhausted from simulated body fluid (SBF) solution very quickly (within hours) under static conditions. In such exhausted SBF solution the formation of Ca–P layer (hydroxyapatite — HAp) was stopped in agreement with the decrease of Ca and P concentrations. On the contrary, highly soluble materials (glass-ceramic scaffold) induced the formation of a new mineral layer also on the walls of the PE container used. For a non-standard shape of the tested materials the usage of dynamic or static–dynamic *in vitro* test arrangement was confirmed to be a better option to test bioactivity. However, also for this type of arrangement it is essential to determine the S/V or S/F ratios (the surface area/volume or flow of SBF solution) very precisely. For detailed understanding of the interaction between the tested material and SBF it is important to analyze the leachates (monitoring Ca^{2+}, $(PO_4)^{3-}$ and minor element concentrations) and to monitor the pH value. An expected result of the *in vitro* test (according ISO standard) is the formation of HAp on the surfaces of tested samples in SBF. However, the formation of hydroxyapatite may not be the proof of their potential bioactivity necessarily (*e.g.* due to the use of TRIS buffer).

1. Introduction

In vitro testing seems to be an ideal method to quickly test the chemical stability of newly developed materials in a solution similar to human blood serum. Such materials must be capable of creating a firm bond with bone tissue. Kokubo et al. prepared ISO standard 23317:2014(E) [1]: "Implants for surgery — *In vitro* evaluation for apatite-forming ability of implant materials". While this standard describes the chemical composition of an SBF solution in detail, certain critical aspects of *in vitro* tests need to be resolved in the near future:

1. SBF
   To simulate the conditions in living organisms, it is very important to use a solution that contains the inorganic part of blood serum. The ISO standard prescribes an acellular and protein free solution; namely, simulated body fluid buffered with TRIS tris-(hydroxymethyl) aminomethane [1]. However, we found [2] that, for example, TRIS buffer used in SBF supports the dissolution of the crystalline phase of glass-ceramics and the crystallization of HAp on its surface, thereby leading to false positive *in vitro* test results. Bohner and Lemaitre [3] similarly note that "*the use of SBF for bioactivity testing leads to the positive and false negative results*".
2. Limitations to the bulk compact shape of an inorganic sample
   The description of the form and dimensions of test samples given in the ISO standard is limited to compact samples with a defined S_a/V ratio (sample surface area/ SBF volume). Rules for materials in the form of the grit, scaffolds and granules (with a large surface area) used in ordinary clinical practice (*e.g.* HA, TCP, glass-ceramics) are missing.
3. Arrangement of *in vitro* test
   The ISO standard only covers the static arrangement of an *in vitro* test, in which a limited volume of solution (SBF) is used and not changed during the test. Under such static conditions, the biogenic elements (Ca or P) of SBF can be quickly exhausted. However, inside a real living organism, SBF is never exhausted. Therefore, to more closely resemble the conditions in an *in vivo* environment, the dynamic arrangement of an *in vitro* test has been used [4–7].
4. HAp formation
   The ISO standard [1] requires that the *in vitro* test results in the formation of hydroxyapatite on the surface of tested material. However, this may not be the sole indicator of the behavior of the material in the human body [8]. Ion release may be the other appropriate indicator of the reactivity of a tested material.

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5. The term "bioactivity"
   It has been used in a major part of the published works, in which the material is tested only *in vitro*, probably because of the convenience of the single word expression. In fact the term "bioactivity" should be used only in connection with *in vivo* tests [9]. When discussing results of *in vitro* tests, as carried out in the present study, the term "reactivity" appears as more appropriate.

In this work, under various conditions, we perform *in vitro* tests on silicate glass-ceramic scaffolds derived from 45S5 glass and calcium phosphates (HA, β-TCP), and interpret results in relation to the aforedescribed issues.

2. Experimental procedures

2.1. Materials

2.1.1. Glass-ceramic material

The silicate material used for testing was the a glass-ceramic (45S5 bioactive glass based) in the form of a highly porous structure (scaffold) prepared by the foam replica technology [10]. The material contained crystalline and residual glass phases as shown in Table 1.

2.1.2. Calcium phosphate materials

The tested materials (white granules, 1–2 mm) were prepared by Lasak Ltd., Prague, Czech Republic. The first material – resorbable β-tricalcium phosphate (Poresorb®-TCP, β-$Ca_3(PO_4)_2$), called TCP with the specific weight 2900–3100 $kg \cdot m^{-3}$ – contained macro (100–200 μm) and micro (1–5 μm) pores. The second material was micro and macro porous hydroxyapatite (OSSABASE®-HA, $Ca_{10}(PO_4)_6(OH)_2$) called HA [11].

2.2. Solutions for the in vitro test

Modified simulated body fluids used for the tests are in Table 2.

2.3. Arrangements of the tests

The arrangements of the *in vitro* tests are presented in Table 3.

2.4. Analysis of the materials

2.4.1. Scanning electron microscopy/energy-dispersive spectroscopy (SEM/EDS)

The surface of tested materials before and after the tests was inspected with a Hitachi S-4700 scanning electron microscope (SEM) equipped with an EDS analyzer (NORAN D-6823) working at an accelerating voltage of 15 kV. The tested materials were powder coated with an Au–Pd layer during 80–100 s for SEM observations.

2.4.2. X-ray powder diffraction analysis (XRD)

The tested materials were ground in an agate mortar in a suspension with cyclohexane. The suspension was then placed on a mylar film and fixed to a transmission sample holder. After solvent evaporation a thin layer of the prepared sample was covered with another mylar film.

Table 1
Compositions of 45S5 bioactive glass and individual phases of the 45S5 bioactive glass based scaffold (wt.%) [10].

Oxide	45S5 (100 wt.%)	$Na_2O \cdot 2CaO \cdot SiO_2$ (77.4 wt.% of scaffold)[a]	Residual glass phase (22.6% of scaffold)
SiO_2	45.0	50.9	24.8
Na_2O	24.5	17.4	48.5
CaO	24.5	31.7	–
P_2O_5	6.0	–	26.5

[a] The minority crystalline phases $CaO \cdot SiO_2$ and $NaCaPO_4$ (buchwaldite) are included.

The diffraction patterns were collected using a PANalytical X'Pert PRO diffractometer equipment with a conventional X-ray tube ($Cu_{K\alpha}$ 40 kV, 30 mA, line focus) working in the transmission mode. An elliptic focusing mirror with divergence slit 0.5°, an anti-scatter slit 0.5° and a soller slit of 0.02 rad were used for the primary beam. A fast linear position sensitive detector PIXcel with an anti-scatter shield and a soller slit of 0.02 rad was used for the diffracted beam. All patterns were collected in the range of 3 to 88° 2 theta with the step of 0.013° and 200 or 600 s per step producing a scan of about 4.5 h. Qualitative analysis was performed with a HighScorePlus software package (PANalytical, the Netherlands, version 2.2.5 or 3.0 e), a Diffrac-Plus software package (Bruker AXS, Germany, version 8.0) and JCPDS-ICDD PDF-2 database.

2.5. Solution analysis

All tests and analyses were measured using two parallel series of samples.

2.5.1. Atomic absorption spectrophotometry (AAS)

The concentration of Ca^{2+} ions in the leachate from all types of solutions was analyzed with a VARIAN-Spectr AA 300. The so-called release agent (KCl) was added to each sample to determine the quantity of Ca. Atomization was performed in acetylene-N_2O flame. The wavelength used for absorbance measurements was 422.7 nm.

2.5.2. Spectrophotometry

The concentration of $(PO_4)^{3-}$ ions (except mod-DMEM) was analyzed with a UV–VIS Spectrophotometer UV1601 at $\lambda = 830$ nm (ČSN 830540). Ion concentrations were calculated using a calibration line method from the measured absorbance values.

2.5.3. Inductively coupled plasma-optical emission spectroscopy (ICP-OES)

The concentration of P in mod-DMEM leachates was measured by ICP-OES with a PerkinElmer-Optima 2000DV instrument. The leachate was vaporized with a Gem-ConeTM nebulizer and the flow rate of the leachate through the nebulizer was 2.2 $ml \cdot min^{-1}$. The produced fine aerosol was carried with an argon stream into a plasmatic burner (1300 W). The concentrations were measured at wavelengths 231.620, 214.917 and 178.221 nm.

2.5.4. pH measurement

The pH in all types of leachates was measured with an inoLab pH-meter with a combined glass electrode at the laboratory temperature (dynamic test) and at 36 ± 1 °C (static and static–dynamic test).

3. Results and discussion

3.1. Solutions for the in vitro tests

The effort to define a relation between *in vitro* and *in vivo* tests has resulted in development of testing solutions which simulate human cellular fluid. To determine the ability to form apatite on the surface of glass or glass-ceramics, some authors [12–14], have used TRIS buffer alone, which is well known from biological laboratories. A turning point in the development of solutions simulating *in vivo* conditions were the solutions prepared by Kokubo et al. [15] and also by other authors [16]. In the mentioned studies, in addition to TRIS buffer (pH = 7.25 at 36.5 °C), the simulated solutions also contained ions in concentrations close to the inorganic part of blood plasma, however the concentration of HCO_3^- was only 4.2 $mmol \cdot l^{-1}$ instead of 27 $mmol \cdot l^{-1}$. Höland, Völksch et al. [16] explained the apatite phase formation on the A-W glass-ceramics by reaction of the material and by the effect of ions from the simulated solution. In 2001 Helebrant et al. [17] and later Müller and Müller [18] tested a series of SBF solutions with gradually increasing concentrations of HCO_3^- ions, up to the value close to blood plasma. They found that carbonate hydroxyapatite

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