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Production and characterization of a glass-ceramic biomaterial and in vitro and in vivo evaluation of its biological effects

Bir cam-seramik biyomalzemenin üretimi, tanımlanması ve biyolojik etkilerinin canlı-dışı ve canlı-içi ortamda değerlendirilmesi

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Amaç: Cam-seramikler kemik yerine kullanılabilen, genellikle sol-jel yöntemiyle elde edilen biyomalzemelerdir. Kemik dokusu ile organik bağlarla bütünleşmesi (osteoentegrasyon) önemli özellikleridir. Bu çalışmada bir biyocam-seramik üretilerek, yapısal özellikleri ve canlı-dışı (*in vitro)* ve canlı-içi (*in vivo)* biyolojik etkileri değerlendirildi.

Çalışma planı: Sol-jel toz sentezi yöntemiyle, tetraetilortosilikat, dibütilfosfat, magnezyum ve kalsiyum nitrat kullanılarak, $30SiO_2$-17MgO-$53Ca_3(PO_4)_2$ formülüne yakın cam seramik elde edildi. Örneklere 1100 °C'ye kadar sıcaklık uygulanarak, mikroyapıları ve oluşan kristal fazlar taramalı elektron mikroskobu ve X-ışını kırınımı (difraksiyon) (XRD) ile incelendi. Canlı-dışı test için, cam-seramik örnekleri 10, 30 ve 40 gün süreyle, plazma içindeki iyonları içeren yapay vücut sıvısı (YVS) içinde bekletildi. Daha sonra, XRD ile incelendi. Son olarak, canlı-içi test için örnekler Sprague-Dawley türü sıçanların tibia kemiklerine gömülerek kemik dokusu ile 4, 6 ve 8 haftalık sürelerde bütünleşmesi incelendi.

Sonuçlar: Üretilen cam-seramikte sıcaklık artmasıyla kristal fazların büyüdüğü görüldü. Yapay vücut sıvısı içinde 10 gün bekletilmiş örneklerde XRD'de değişiklik olmazken, 30 ve 40 gün bekletilen örneklerin 2. ve 3. derece kristal evrelerinde hidroksiapatit kristal oluşumu gözlendi. Canlı-içi deney sonuçları, cam-seramiğin kemiksi dokunun yerini almaya ileri derecede yatkın olduğunu ve sekiz hafta içinde kemik ile bütünleştiğini gösterdi.

Çıkarımlar: Ürettiğimiz cam-seramik yüzey-reaktiftir ve ortopedide kemik yerini tutucu malzeme olarak kullanılabilir.

Anahtar sözcükler: Biyouyumlu malzeme; seramik; cam; toz; sıçan; yüzey özellikleri.

Objectives: Glass-ceramics are biomaterials that are usually produced by the sol-gel technique and can be used as a substitute for bone. One important feature of glass-ceramics is osteointegration with bone tissue. This study was designed to produce a glass-ceramic and evaluate its structure and *in vitro* and *in vivo* biological effects.

Methods: With the sol-gel method, a glass-ceramic was synthesized in the form of $30SiO_2$-17MgO-$53Ca_3(PO_4)_2$ using tetraethylorthosilicate, dibutyl phosphate, magnesium, and calcium nitrate. Glass-ceramic jel samples were sintered at temperatures up to 1100 °C and their microstructure and phases were examined by the X-Ray diffraction (XRD) technique and scanning electron microscopy. For *in vitro* tests, the samples were immersed in a simulative body fluid (SBF) for 10, 30, and 40 days to be analyzed by XRD. For *in vivo* tests, the samples were placed in tibial metaphyses of Sprague-Dawley rats for 4, 6, and 8 weeks for histological evaluation of osteointegration.

Results: As the temperature increased, growth of crystal phases was noted. While XRD analysis showed no change in samples that were kept in SBF for 10 days, hydroxyapatite crystals were seen after 30 and 40 days of SBF treatment in the second and third degree of crystal phases. In vivo test results showed that the glass-ceramic possessed a high tendency to replace osteoid bone tissue, with full osteointegration at eight weeks.

Conclusion: The glass-ceramic produced has a high surface reactivity and can be used as a bone substitute material.

Key words: Biocompatible materials; ceramics; glass; powders; rats; surface properties.

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The ceramics, comprising a major part of the biomaterials, can be classified according to their live tissue attachment as inert (Al2O3), resorbed (Ca3PO4, hydroxyapatite) and surface-reactive (glass-ceramic, glass). Surface-reactivity is the ability to transfer Ca, P elements to the surface and form the HA (hydroxylapatite) crystal. Surface-reactive ceramics are generally fabricated by melting, ceramic processes or the sol-gel method, where sol-gel stands for the first two letters of the "solution-gelation" processes. In other words, it is a method of solution-gelation. In the sol-gel technique, mainly various metal alcosytes, metal salts, alcohol and organic solutions and water are used, and it is the most convenient production method for surface-reactive ceramics to be used in powder. The grain size of the crystal phases of the powder can be caught only in a nanometer scale. All these characterizations can be achieved by both the productive and post-productive heating processes. The glass ceramics are harmles to the body, integrating with the bone tissue when they are implanted into the live bones. This is also an indication of their biocompatibility.

Due to hardness and less abrasion, hard ceramics are used for head and acetabular cups of prothesis. It is also likely to use them in powder as a substitute material for bones. Such ceramics should be easy to produce and have a controllable production process. Many local ceramic scientists as well as their foreigner collegaues are concerned about the biological effects of the material they produced. The produced material must be tested in vitro with plasma-like fluids and several cells such as fibroblast, osteoblast, and also in vivo in animal models to determine the superiority and diversity of its interaction with the tissue. It is essential for the producer to describe the characteristics and biological effects of the material.

The discipline of "Biocompability Research" is a rarely studied field in our country. The main feature of the present study was that the biocompability of the material was studied with a team experienced in surgery and the bone tissue, including a multidisciplinary approach.

The aim of the present study was to produce and characterize glass and glass-ceramic powders by the sol-gel process with a formula of 30% SiO2-17MgO-53Ca3PO4 for use in the orthopedic surgery and also analyse their in vitro and in vivo biological effects.

Material and method

Preparation of Glass Ceramics

In order to prepare the glass and glass-ceramic in the target structure of 30% SiO2-17MgO-53Ca3PO5; TEOS (tetraethylorthosilicate), dibutylphosphate (C12H27O4P), and Ca-nitrate (CaNO3) and Mg-nitrate (MgNO3) salts were used as sources of SiO2, P2O5SiO2 and MgO and CaO, respectively. Furthermore, a solution was prepared using distilled water, hydrochloric acid and acetic acid. As a result of hydrolysis and polimerization reactions, the solutions transformed into gel. Then, a clear solution prepared at the room temperature using a magnetic mixer, was kept in incubator at a temperature of 40ºC to achieve the gelation process after gradual drying. The calcination of the gel was achieved by sintering it in an electrical oven at temperatures 100, 500, 900 ve 1100ºC for one hour, and afterwards leaving it to cool down. The calcinated gel samples were softly pounded to powder, and they were used in all characterization procedures and in vivo and in vitro tests. The calcination is performed by heating, which provide diversification and multiplication of the crystal structure in ceramic, and then the crsytal structure disappears after induction of excessive heats above the limits -1400° C for our sample- to form the glass.

Characterization

During characterization, for chemical analysis, semi-quantitative X-Ray-Flourescence Spectrometer (XRF, Philips, PW 2404 WDXRF); for phase analysis, X-Ray Diffraction (XRD, Shimadzu, XRD-6000), and for micro-structural examinations and analyses, Scanning electron microscopy (SEM-EDS, JEOL, SEM-6335F) were used.

The XRD analyses in the glass-ceramic powders were carried out at where 2 teta scanning angles had an interval of 0-70º.

The mechanical properties of the powder glass-ceramic were not studied.

In-vitro test method: A Simulated Body Fluid (SBF) including equal weights of the ions (Na+142,K+5,Ca++2.5,Mg++2,Cl-147.8,HCO3-2,HPO-1,SO4-5, as mol mm-1) in the plasma was prepared for use (Biofarma® /Türkiye). The samples derived from the glass ceramics and calcinated at 100, 500, 900 until 1100ºC were kept in the Simulated Body Fluid (SBF) for 10, 30 and 40 days. Then, they were analyzed by XRD to determine the crystal changes and crystals including Ca, P and HCA.

Table 1. Aimed and actual formula results in the chemical analysis of ceramics with XRD flouroscopy.

Compound	Aim (% Weight)	Measured (% Weight)
SiO_2	30	28
MgO	17	15
CaO	28.73	38
P_2O_5	24.27	18

In-vivo test method: Based on the appropriate permission relevant to the study protocol for the Experimental Animal Breeding and Cultivation Center of the Medical Faculty of the Cerrahpaşa University, the glass ceramic samples processed by the sol-gel technique and calcinated at 900ºC were implanted into the proximal tibial metaphysis of the right and left extremities of the Sprague Dawley rats in groups of five. To do that, samples with more surface reactivity and the most hydroxyapatite crystals were selected following the heat treatment at 900ºC and immersion in SBF. The ceramic powder was placed onto areas of 1mm3 (1x1x1) by fine carving tips in ceramic. The proximal tibial metaphyses of the rats sacrified after weeks 2, 4, 6 and 8 were surgically removed. The bones with implants were fixed in the buffered formalin solution, and following decalcification by formic acid, they were dehydrated with increasing degrees of alcohol, and embedded in paraffin, and then prepared in sections of 5-10 mm thick, and stained with Hemotoxylin-Eosin (H-O) followed by an analysis of light microscopy (Bx50 Olympus).

The interaction of the glass ceramics dried keeping in sections at 100ºC with the osteoid tissue of live bones was analyzed over time.

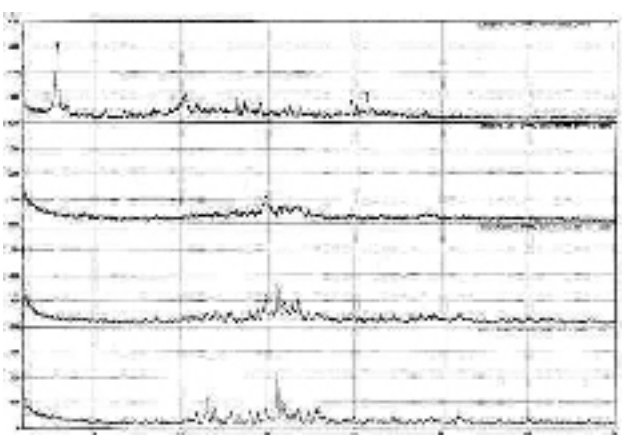

Figure 1. X-Ray Difraction diagrams of glassceramics heated at different temparatures.(100,500,900,1100).

Table 2. Chemical distribition of main components of ceramic samples in heated at 500 C in XRD fluoroscopy

Oxide	% Weight
SiO_2	34.15
MgO	18.59
CaO	32.57

Results

A post-production chemical analysis by XRF showed deviations from the target formula. The target and actual formulas defined by XRF are shown at Table 1. As shown in the table, the deviations usually occurred in the CaO and P2O5 components, in which the chemicals initially used might have played a role. As the objective was not to obtain a completely cytometric formula in this production, the glass ceramic formula we obtained was suitable for the aim of the study. The XRD studies on the the glass-ceramic powders were performed with a scan angle of two teta ranging from 0 to70º. The post-calcination XRD results are shown at Figure 1. With increase in calcination temperature in the glass-ceramic samples, a growth was observed in the size of the XRD peaks (Figure-1). The phases described according to the XRD diagrams are shown at Table 2.

Micro-structural and elemental analyses by SEM showed that the distribution of elements in the system was highly homogenous and the gel powders were generally consisted of very small crystals in nanometric

Table 3. Defined phases according to X-ray difraction diagrams.

Temperature (°C)	Phases
100	1. Saponite-$Ca_{0,2}Mg_3(Si, Al)_4O_{10}(OH)_2.4H_2O$
	2. Monohydrocalcite - $CaCO_3.H_2O$
500	1. Calcite - $CaCO_3$
	2. Monticellite - $CaMgSiO_4$
	3. Paragonite - $Mg_3(PO_4)_2$
900	1. Akermanite - $Ca_2MgSi_2O_7$
	2. Quartz - SiO_2
	3. Paragonite - $Mg_3(PO_4)_2$
	4. Monticellite - $CaMgSiO_4$
1000	1. Akermanite - $Ca_2MgSi_2O_7$
	2. Paragonite - $Mg_3(PO_4)_2$
	3. Monticellite - $CaMgSiO_4$
	4. Quartz - SiO_2
	5. Calcite - $CaCO_3$

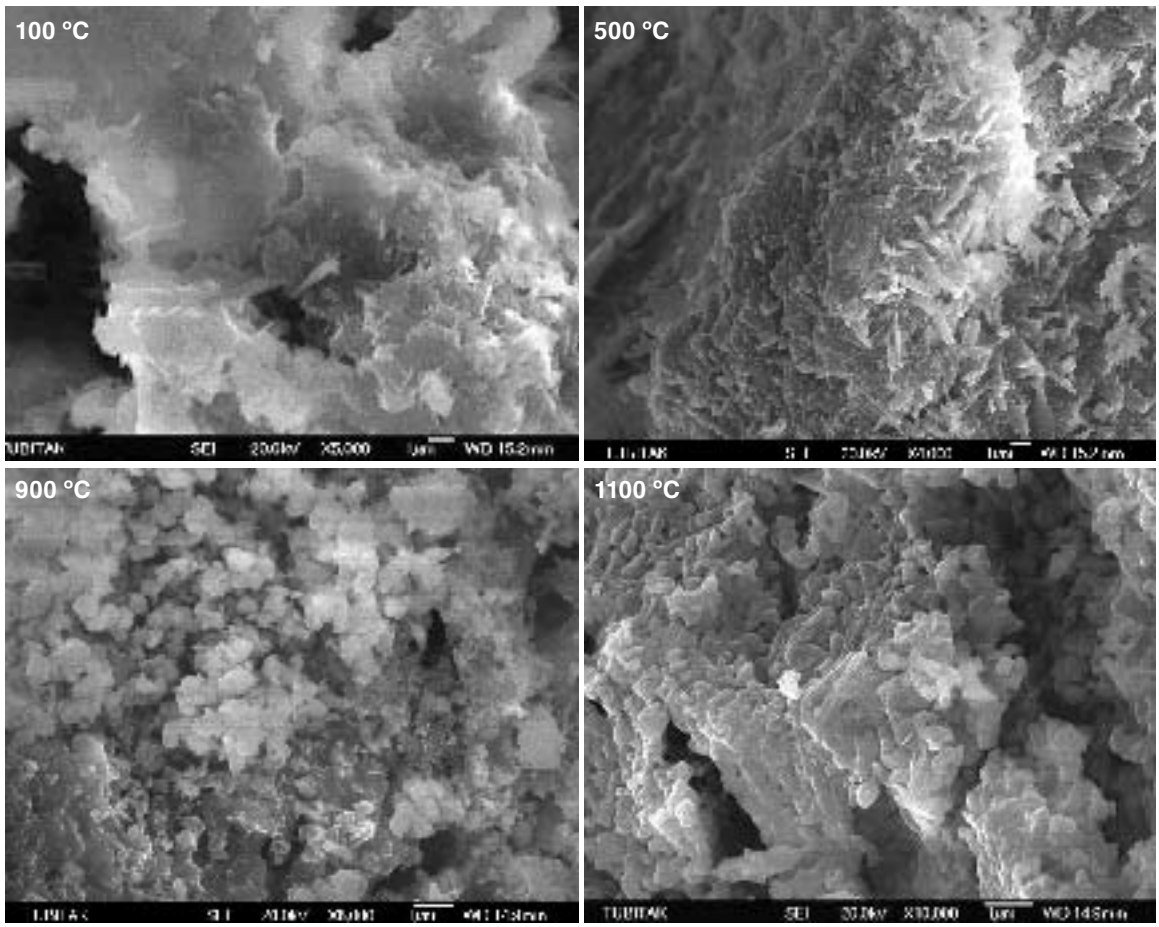

Figure 2. SEM microphotographs of ceramics heated at different temparatures 100º, 500º, 900º,1100º C.

dimensions. No procedure was performed on the material for SEM analysis. The samples, being vacuumed as part of the technique, were studied later on. Analysis of microstructural SEM images indicated that as the temperature increased, new phases emerged or growth of the emerging phases was increased. It was also oberved that various crystals became differentiated (Figure-2 a,b,c,d.).

During the analysis of SEM micro-structures, a loose structure was observed at 100ºC while with the increased temperature, bacilliform structures were formed, and initiation of sintering was observed particularly at 900 and 1100ºC. In both cases, formation of grains/crystals in nanometric dimensions can be mentioned, which means both a wide surface area and a high reactivity.

In-vitro test results

No remarkable changes were observed in the phase formation and micro-structures of the powders kept in SBF (Simulated Body Fluid) for 10 days. This can be explained by the fact that an experimental in vitro period of 10 days was not sufficient for occurence of any reaction. On the other hand, detection of hydroxylapatite crystals not at the main phases, but at the 2nd and 3rd degree phases in the XRD diagrams taken at the similar device adjustments in the samples which were kept for 30 to 40 days is an indication of the exchange of Ca, P elements on the particule surfa-

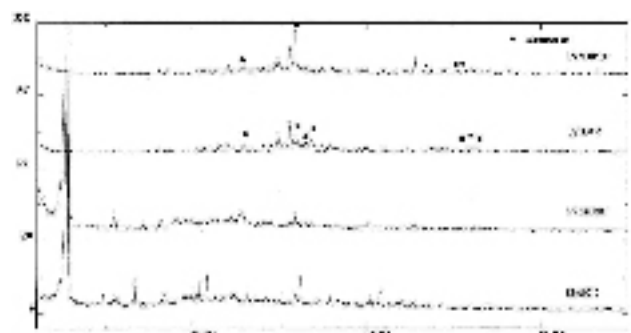

Figure 3. Comparative demostration of XRD changes after being heated at 100,900 ºC and treated with simulative body fluid.

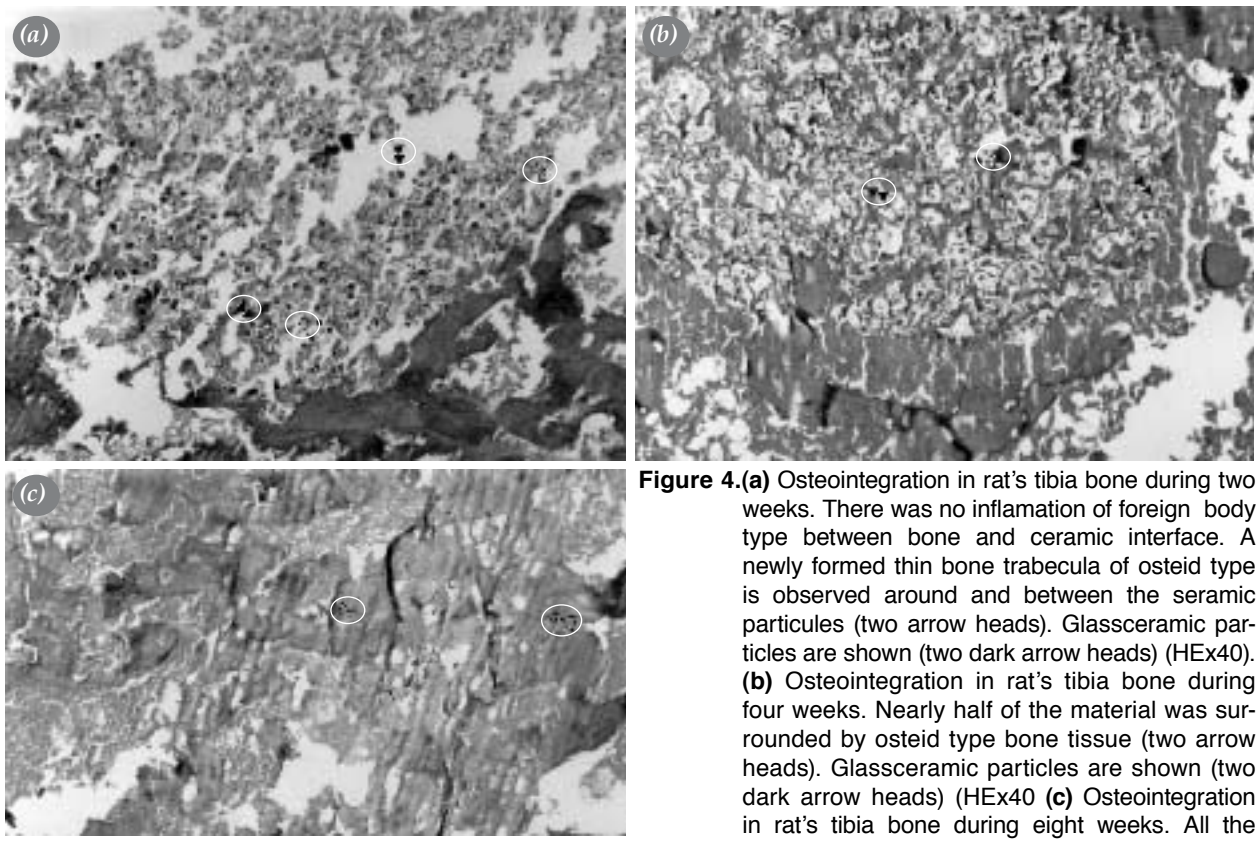

Figure 4.(a) Osteointegration in rat's tibia bone during two weeks. There was no inflamation of foreign body type between bone and ceramic interface. A newly formed thin bone trabecula of osteid type is observed around and between the seramic particules (two arrow heads). Glassceramic particles are shown (two dark arrow heads) (HEx40). **(b)** Osteointegration in rat's tibia bone during four weeks. Nearly half of the material was surrounded by osteid type bone tissue (two arrow heads). Glassceramic particles are shown (two dark arrow heads) (HEx40 **(c)** Osteointegration in rat's tibia bone during eight weeks. All the material was invaded by osteid bone tissue of lamellar type (two arrow heads). (HEx100).

ces and formation of HA crystals, which means that the glass-ceramic is surface reactive (Figure- 3).

Although it forms more stable crystals at higher temperatures of 900ºC, the glass-ceramic exchanges ions, which is an indication of its surface reactivity (Figure-3).

The glass-ceramics form more stable rod crystals at 900ºC, resulting in novel hydroxyapatite crystals by exchanging ions after treatment with YVS, and that is an indication of more surface reactivity (Figure-3) , and also our main rationale using the in vivo model.

In-vivo test results

The sections obtained 14 days after the implant showed presence of the thin immature bone (osteid) trabeculae attached to the material. No inflammatory reaction against any potential foreign body and fibrous tissue were observed between the glass-ceramic material and the thin lamellar bone tissue (Figure-4a). As a result of histologic analysis, it was found that half of the glass-ceramic material was replaced and surrounded by the lamellar and spongy type of bone (Figure-4b). At the day 60, the entire material implanted was replaced by a lamellar bone tissue (Figure-4c). It can be indicated that the pseudoeosinophilic appearance of the bone marrow in each section resulted from the declacification by strong acid for a long period of time. One of our significant results was the augmentation, and expansion of the osteoid tissue over time, and absence of any fibrous tissue in between.

Discussion

The sol-gel technique is one of the most convenient methods for powder synthesis in order to control the size, shape and surface area of the grains, i.e. controlled features. As it is studied at the room temperature under very simple conditions, it is a very practical, fast and economic method and it succesfully achieves synthesis of the compositions, which are very complicated to be produced by conventional methods as it provides the mixture at a molecular level. Its biological characteristics can also be controlled as it can produce powders of any size and morphology as well as the of chemical purity. The morphological features such as granule or

powder forms can alter the biological effects of glass-ceramic.

High porous ceramics can be obtained by the sol-gel technique, with a more bioactive synthesis of the glass compared to the one obtained by the melting method. [1,2]

As seen in Table-1, the deviations usually occurred at the compounds CaO and P2O5. Here, the initial chemicals might have played a role. The glass-ceramic formula was found convenient for the present study as the objective of this production was not to obtain an entirely stoichiometric formula.

The mechanical tests were excluded as the material was in powder and no mechanical benefits were expected in use although it was essential for the characterization of the material. Furthermore, the mechanical properties can be studied when required.

In order to obtain the ceramic by the sol-gel method, heat application, i.e. thermal decomposition (calcinations) is required to remove the organic wastes, gas products and water molecules. In the present study, procedures with increasing heating temperatures were used for the crystallization of the glass-ceramics prepared by the sol-gel technique. The duration for the heating procedures carried out in the electrical ovens was one hour.

The sample diagram analyzed at 100º C showed an initial higher peak, however it disappeared in the following gradually increasing heats. It is associated with the organic solvents inside the ceramic.It was found that with increased temperature, the crystal phases inside the glass-ceramic structure are grown and enhanced, and its boundaries become more remarkable (Figure-1).

The results of the studies on glass and glass-ceramics including bioactive silica support that the SiO2 increases the formation of a surface layer rich in calcium and phosphore due to its solubility in an amorph or crystal phase. [3] The glass-ceramics attach to the live bone tissue, forming hydroxylapatite by exchanging ions when interfaced with the fluid (SBF, plasma) with the Si-OH groups formed in the silica (SiO2) and its surfaces,[4] and furthermore, significance of soluble silica has been reported by many authors, highlighting that the layer rich in silica (SiO2) is an important focus for apatite nucleation.[1,5,6,7] When the solubility of several oxides in the material is high, the formation of surface layer responsible from bioactivity has been facilitated.[3]

Presence of calcium and phosphore in the apatite phase of the glass-ceramic prevents precipitation of the surface apatite.[8]

The surface reactive ceramics are available in several formulas. For an ideal mixture, it should be controlled with easy and quick in vitro tests. The SBF including the ions in the blood plasma is the initially prepared test material, which is a test fluid frequently used in the assay of the surface reactivity of the glass-ceramics. Another in-vitro test is the analysis of the effect of glass-ceramics on the shape and number of the osteblast cells. It was found that in the in vitro culture tests performed using the human osteoblasts, the silicon derived from the bioactive glasses in a critical density (derived from 45S5® glass ceramic by melting at a speed of 15 ppm) organizes the growth factor proteins and increase the osteoblast ingrowth. [9] In the present study, no osteoblastic cell was used.

The fact that glass-ceramic sintered at 900∞C forms hydroxylapatite and other crystals, interacting with the ions in the SBF is an indication of the presence of surface reactivity.

The surface-reactive glass-ceramics have been analysed in detail for potential use as bone grafts due to their attachment characteristics with the live bone. [10] Naturally, glass-ceramics can also attach to the bone.[6,7,11] The glass systems, which were produced in formulas including SiO2-CaO-P2O5-Na2O in natural ratios are the first glass materials with bioactive behaviors.[8,12,13]

For the biocompatibility of materials, some tests are performed in the bone tissues of animals and use in human volunteers. These are particulary required for the clinical trials of the biomaterials.

The in-vivo tests are complicated, time-consuming and expensive, and also require compliance with the ethical principles for animal rights. It is not possible to carry out a new transplantation test for each change in the formula of the glass-ceramic in living subjects. Thus, such tests are held at the final stage.

In the present study, the osteo-integration of the glass-ceramic samples with the rat bone was progressive without any inflammation and fibrous tissue. Another important point was the resistance experienced during the preparation of 2 to 8 week sections when cutting

by microctome. The glass-ceramic material is hardened during interaction with the live bone tissue.

Based on the in-vivo test results, the glass-ceramic we produced have the ability to osteo-integrate with the bone tissue, and it can be potentially used as a biomaterial, as other similar examples around the world.

The hydroxyapatite crystals of ceramic are used in forming the inorganic component of the bone with the activity of the alkaline phosphotase enzyme while the osteoid matrix is formed. The enlargement of the purple stained tissue sections (Figure ..), and reduction in the ceramic granules indicate that more ceramic was used for the structure.

The quantitative and qualitative analysis results of the reactioner cells in the histological analysis of the bone sections are inadvertently non-available. It will be possible to provide the morphometrical and numerical results with a computer-aided microscope only after preparing the sections without decalcifying by Saw-Microtome, supplied by means of a governmental support. We plan to provide the time-based changes by taking sections more frequently, which is subject to another histological research article (ref)

The disadvantages of the bioglass-ceramics we produced include lack of necessary hardness, and lack of elasticity and resistance to heavy load. However, its hardening following interaction with bone after it was implanted, and its integration with the bone without any fibrous tissue is a significant feature of this material.

Conclusions

Following the removal of cysts and tumoral lesions, they can be used as replacement grafts for the bones. When it is prepared compositely in combination with other hard materials, its value will be increased if required much harder. The use of osteoblast cells in in vitro tests should be an additional option in the analysis of the biological efficacy.

References

1. Sepulveda P, Jones JR, Hench LL. Characterization of melt-derived 45S5 and sol-gel-derived 58S bioactive glasses. J Biomed Mater Res 2001;58:734-40.
2. Hench LL, West JK. Biological applications of bioactive glasses. Life Chem Rep 1996;13:187-241.
3. Oliveira JM, Correia RN, Fernandes MH. Surface modifications of a glass and a glass-ceramic of the MgO-3CaO. P2O5-SiO2 system in a simulated body fluid. Biomaterials 1995;16:849-54.
4. Kokubo T, Kushitani H, Ohtsuki C, Sakka S, Yamamuro T. Chemical reaction of bioactive glass and glass-ceramic with a simulated body fluid. J Mater Sci Mater Med 1992; 3:79-83.
5. Pereira MM, Clark AE, Hench LL. Effect of texture on the rate of hydroxyapatite formation on gel silica surface. J Am Ceram Soc 1995;78:2463-8.
6. Ebisawa Y, Kokubo T, Ohura K, Yamamura T. Bioactivity of CaO.SiO2-based glasses: invitro evaluation. J Mater Sci Mater Med 1990;1;239-44.
7. Li P. In vitro and in vivo calcium-phosphate induction on gel oxides [PhD Thesis]. Leiden University, The Netherlands, 1993.
8. Hench LL. Bioceramics: from concepts to clinic. J Am Ceram Soc 1991;74:1487-510.
9. Kokubo T. Surface chemistry of bioactive glass-ceramics. J Non-Cryst Solids 1990;120:138-51.
10. Xynos ID, Edgar AJ, Buttery LD, Hench LL, Polak JM. Ionic products of bioactive glass dissolution increase proliferation of human osteoblasts and induce insulin-like growth factor II mRNA expression and protein synthesis. Biochem Biophys Res Comm 2000;276:461-5.
11. Ogino M, Hench LL. Formation of calcium phosphate films on silicate glasses. J Non-Cryst Solids 1980;38-39:673-8.
12. Vogel W, Holand W. The development of bioglass ceramics for medical applications. Angew Chem Int Ed 1987; 26:527-44.
13. Gross U, Brandes J, Strunz V, Bab I, Sela J. The ultrastructure of the interface between a glass ceramic and bone. J Biomed Mater Res 1981;15:291-305.
14. Ceyhan T, Gülçubuk A, Sayrak H, Karaca Ç. Polietilen glikol tereftalatın biyouyumluğunun in vivo sıçan kemik modelinde histolojik olarak incelenmesi. Eklem Hastalıkları ve Cerrahisi 2007;18:33-8.
15. Bilir A, Ceyhan T, Altinoz MA, Guneri AD, Bayrak I, Altug T. Culturability of osteoblast cells extracted from mature an fetal BALB/c mice calvaria. [Article in Turkish] Acta Orthop Traumatol Turc 2000;34:389-95.
16. Ceyhan T, Bilir A, Karaca C. Culturability of rat bone marrow stromal cells and evaluation for osteoblastic formation. [Article in Turkish] Acta Orthop Traumatol Turc 2006;40:67-71.