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IN-VIVO REACTIONS IN SOME BIOACTIVE GLASSES AND GLASS-CERAMICS GRANULES

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Abstract

Two different bioactive glasses and one glass-ceramic were implanted as granules for 2 months in rabbit muscle and for 5 months in sheep jaw in order to study the influence of the biological surroundings on the reactions of the materials. Scanning electron microscope and energy dispersive X-ray microanalysis showed that a calcium and phosphorous-rich (CaP) surface layer (adjacent to a silicon rich-layer) forms on both glasses in both implantation sites. The glass-ceramic developed only a CaP layer. A chemical process of degradation was more evident in muscle, even though the implantation time was shorter than that in bone. For all materials, a chemical bond with bone occurs. The amount of new formed bone was different for the three materials. Aging of the most reactive glass is hypothesized to explain a rapid loss of silicon by diffusion, as indicated by infrared spectroscopy.

Key Words: Glasses, glass ceramics, bioactivity, implantation, sheep, rabbit, biodegradation, bone interface, X-ray microanalysis, scanning electron microscopy.

Introduction

Bioactive glasses are attractive materials for implantation, since they interact positively with bone and sometimes with soft tissue, developing chemical bonds; but the steps of these interactions are still being studied.

It was first shown by Hench that bone can adhere to bioactive glasses through a chemical bond [12, 13]. In addition to their bone bonding ability, bioactive glasses also conduct bone growth along their surfaces [11, 17, 20]. This osteoconduction is important in bone substitutions such as the filling of bone defects. In this application, granules may prove more useful than blocks. The physico-chemical reactions of glasses and glass-ceramics with bone as bulk specimens are being widely investigated [2, 4, 10, 11, 16, 21]. In contact with the body fluids, the surface of glass is transformed into a Si-rich layer into which calcium and phosphorus ions, coming from both the biological fluids and the glass, penetrate and precipitate as calcium-phosphate [1, 8, 19]. This leads to the formation of an apatite surface layer to which bone can bind. For bioactive glass-ceramics, it has been reported that the apatite surface layer forms without the SiO₂-rich layer [7]. When using glasses or glass-ceramics as granules, other properties must be considered [5, 6]. The interfacial reactions as well as the biological response may be different due to a local high surface-area / solution volume (SA/V) ratio. To use these materials as fillers, it is important to understand the reactions of the granules and the effect of a high SA/V ratio.

Since the biocompatibility of these materials is already widely demonstrated [9, 12, 16], the present work presents the behaviour and the possible degradation of two bioactive glasses and one glass-ceramic in bone and muscle with the aim of comparing the superficial morphologies and the reaction mechanisms for granules.

Materials and Methods

Materials

The glasses used were:
"Aged" Bioglass® (registered U.S. trademark, University of Florida, Gainesville). This 45S5 Bioglass® was produced according to Hench's formula in 1987 by American Biomaterials Co. (Plainsboro, NJ), USA, crushed to granules, and stored for 5 years in a so-called "air-tight" box in a laboratory where the temperature ranged from 18 to 35°C and the humidity ranged from 50 to 70%. Since early results for this glass were not consistent with those found previously by Hench and collaborators [12, 13], some analyses were carried out to understand this behaviour. The analyses are reported below.

SS3P4 glass (by Andersson, Finland).

A-W glass-ceramic (by Yamamuro and Kokubo, Japan). The aging of the melted glasses and the storage period following crushing did not exceed 1 year from the preparation of the glass-ceramic.

Table 1 shows the nominal composition of the three materials. Granules (300-500 µm size) were placed in a 4-mm diameter syringe and ethylene oxide sterilized.

Methods

The experiment was divided into two parts:

a) Implantation in 4-mm holes surgically drilled in sheep's mandible for 5 months;

b) Implantation in pockets surgically created in rabbit's dorsal muscles for 2 months.

After sacrifice the bone or the biological tissue containing the granules were explanted, fixed in 4% paraformaldehyde and embedded in methylmethacrylate resin. Sections were cut with a diamond saw (Accutom (empty); the solid) and from the area at point "l" of Figure 2a (solid) and from the area at point "1" of Figure 2a (empty); the Ca/P count ratio has changed from 5.5 to 1.7.

Figure 2 represents the BSE micrograph (a), and the SE image and X-ray dot maps (b) for Bioglass® granules implanted in a sheep's mandible. A few granules (G) are still present. Few of them are in contact with bone and the amount of newly-grown bone (B) is small. As shown for the rabbit muscle (see above, Fig. 2), the BSE image (Fig. 3a) shows that the external part is brighter, thus it has a higher density than that of the core which is empty. The X-ray dot maps (Fig. 3b) show almost total absence of silicon; it must have been lost into the surrounding biological tissues and transported away by the interstitial fluids. The amounts of phosphorus and calcium have increased (see arrows), and their concentrations are greater than in bone. In the original glass, the amounts of calcium and phosphorus were lower (as shown in Fig. 1b). A different behaviour is shown by SS3P4 granules implanted in muscular tissue (Fig. 4a). In this case, the diffusion of silicon and calcium are different. There is an undegraded core with an adjacent Si-rich layer (Fig. 4b). Externally, a Ca,P-rich layer is present. The spectra (Fig. 4c) from the undegraded core (point 1) (solid) and in the external layer (point 2) (empty) indicate that the Ca/P count ratio has changed from 8 to 1.7. Occasionally large precipitates of calcium-phosphate or hydroxyapatite are found (arrow in Fig. 5) on top of the Ca,P-rich layer.

A similar behaviour is shown by the granules im-

Table 1. Compositions of glasses in weight per cent.

<table>
<thead>
<tr>
<th></th>
<th>SiO2</th>
<th>CaO</th>
<th>Na2O</th>
<th>P2O5</th>
<th>MgO</th>
<th>CaF2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioglass</td>
<td>45</td>
<td>24.5</td>
<td>24.5</td>
<td>6</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>SS3P4</td>
<td>53</td>
<td>20</td>
<td>23</td>
<td>4</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>A-W</td>
<td>46</td>
<td>44.6</td>
<td>16.3</td>
<td>4.6</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>

Bone). Comparing SEM images of the implanted granules with elemental maps shows that in both sites there are similar degradative processes (more evident in muscle, although the implantation period was shorter).
planted in sheep’s mandible (Fig. 6a,b). The amount of new bone is greater than that developed in contact with Bioglass® granules in the same situation. The Ca-P external layer seems to act as a cementing line with the bone. In this case, the amounts of Ca and P are greater than those in the original glass, and also greater than those in the bone (Fig. 1b). It had been noted that when bone grows on the granule’s surface, the ion diffusion is delayed and the degradation of the interface is stopped [9]. The few granules still remaining in the connective tissue present findings similar to those for the granules implanted in rabbit.

Figures 7a-c and 8a,b show the A-W glass granules implanted respectively in muscle and in bone. There is a morphologic peripheral degradation of the A-W granules, especially of those surrounded by connective tissue. The surfaces of the sectioned granules are more fragmented than original granules and other glasses. Many pores can be seen. This degradation is more evident in the granules implanted in muscle than in bone.

In both sites of implantation, the Si-rich layer mentioned above is not evident, as has been shown previously by Kokubo [15]. At the surface of the granule (point 2), the concentration of phosphorus increases relative to the original concentration (point 1) (spectra in Fig. 7c: solid = point 1, empty = point 2). Calcium concentration does not rise very much and is less than phosphorus. The Ca/P count ratio changes from 26.5 to 2.75. However, an external layer, rich in calcium and phosphorus, can be seen (arrows in Fig. 8b), even if the amount of new bone was smaller than that developed by S53P4. Magnesium present in the original composition decreases slowly at the interface. The A-W granules are chemically bonded to the bone, when bone trabeculae come in contact with granules.

**Infrared spectroscopy of 45S5 Bioglass®**

A fresh 45S5 Bioglass® sample and a sample of the 45S5 Bioglass® after a 5-years aging were analyzed using a Fourier-transformed IR spectroscopy system.

Figure 9 shows the IR spectra for 300-500 µm granule samples of 45S5 Bioglass® obtained: (a) after 5-years from preparation and milling; (b) fresh; and (c) after storage for 6 months.

The IR peak at 1075 cm⁻¹ of the fresh Bioglass® (Fig. 9b) splits into two peaks in the Bioglass® stored for 5 years (Fig. 9a). Also the peak at 900 cm⁻¹ has increased substantially in the aged glass. After a 6-month storage (Fig. 9c), there is a small, but meaningful change in both 1075 cm⁻¹ and 900 cm⁻¹ peaks. The 1075 cm⁻¹ peak is due to the primary Si-O-Si stretching mode of the glass network. Changes in these modes show structural deterioration of the network which will accelerate stage 1 (ion exchange) and stage 2 (network dissolution). This leads to rapid release of silicic acid (see refs. 14 and 18 for additional discussion of these reaction mechanisms of glasses). Increasing the surface area to volume (SA/V) ratio by forming granules (SA increases) and implantaing in soft tissues, such as muscle, where motion removes the dissolution products, greatly accelerates glass network dissolution [3]. The SA/V effect is very important when the glass network has < 50% SiO₂, because of the presence of Si atoms with less than 2 bridging oxygens/tetrahedron [3]. The surface reaction with H₂O leads to changes in the Si-O-modifier (Ca, Na) vibrational modes at 900 cm⁻¹ (Figs. 9a, c).

Thus, these findings show that aging of glasses of < 50% SiO₂ in humid air can lead to dissolution in tissues due to the effects of SA/V on ion exchange and network breakdown. Aging occurs within one year and it could be a parameter to take into consideration in quality assurance standards since it can influence the rate of reactivity of bioactive implants, especially in granular form.

**Discussion**

The reactions occurring in glass granules positioned in muscle resemble those in bone [1, 8, 10, 12, 15, 16]. The ions diffusing from and into the granules interact with the ions of the biological fluid involved in the reparative process. For glasses with a high rate of diffusion, such as Bioglass®, the surface rapidly reacts, there is an exchange of ions, and a Ca-P rich layer forms very quickly [21]. These hydrated layers are weak and easily cracked. Along these canals, the degradation can continue, but consists primarily of the passage of silicon toward the external biological tissues. Thus, the core is depleted while the calcium-phosphate surface is stable. With sufficient time (5 months), small granules are gone. Similar findings should be expected in other glasses with a similar composition (< 50% SiO₂).

The S53P4 granules, with a higher SiO₂ content, show a different behaviour from that discussed above. The reactions at the glass surfaces result in a SiO₂-gel layer at the surface from which a Ca-P layer forms; this layer is stable with few fractures. No degradation of the internal part is to be seen. The precipitates found on the surface of S53P4 granules in muscles seem to be associated with formation of the Ca,P-rich layer. This phenomenon was already seen in *in-vitro* tests [1]. Thus, the Ca,P-rich layer forms in the SiO₂-gel matrix of the S53P4 glass and not at the surface of the granule. In the *in vitro* test, the calcium-phosphate precipitates have a heterogeneous consistency and morphology.

The A-W glass-ceramic material, being less reactive [15, 16], shows the same diffusion mechanism in both implantation sites, with a higher rate in muscle than in bone. The diffusion occurs principally toward the biological tissues and the Ca,P-rich layer formed is due to surface reactions incorporating Ca and P from body fluids [15]. This layer is thinner than those in the other glasses.

The bioactivity of all three materials is due to a chemico-physical process, i.e., calcium-phosphate formation, which is the same in both sites of implantation, but with minor differences due to a more reactive environment in the soft tissue. (There is only one difference
for S53P4 which shows a further Ca-P precipitation on the surface). The chemical bond with bone is ensured by the compatibility with the Ca,P-rich layer. The formation of this layer is a necessary, though not a sufficient condition, to obtain the bond with bone (see behaviour of Bioglass®). Nothing can be said about the relationship between chemical degradation and new bone growth or the rate increase in bone growth.

The authors conclude that bioactivity is a matter of proper composition, and a consequence of the solubility of the implants. Another important factor may be the aging of the material. The aging of materials is well known, especially in polymers, but is previously unreported in bioactive glasses. Infrared (IR) analyses showed that changes of some chemical bonds occur during aging that probably alter the structure of the glass.

Figure 1. (a) SE micrograph of original Bioglass® granules (G) with pieces of sheep’s bone (B) (bar = 100 μm); and (b) the X-ray dot maps (clockwise from top-left) for Na, P, Ca, and Si.

Figure 2. (a) BSE microphotograph of Bioglass® granules implanted in rabbit muscle (bar = 100 μm); (b) X-ray dot maps (clockwise from top-left) for P, K, Si, and Ca; and (c) X-ray spectra collected from point "1" in Figure 2a (empty) and from a granule of Fig. 1a (solid).
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Figure 3. (a) BSE microphotograph of Bioglass® granules (G) implanted in sheep mandibular bone (B) (bar = 100 µm); and (b) the same in secondary emission mode (top left) with the X-ray dot maps (clockwise from top-right) for P, Ca, and Si.

At the moment, the authors do not know if this difference is significant in such a way as to compromise the bioactivity of granules in living tissues, but they conclude that the effects of aging of bioactive materials need to be investigated.

Acknowledgments

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References


Figure 4. (a) SE micrograph of S53P4 granules implanted in rabbit muscle (bar = 100 µm); (b) X-ray dot maps (clockwise from top-left) for Si, Ca, K, and P; and (c) X-ray spectra collected from points 1 (solid) and 2 (empty) in Fig. 4a.
Figure 5. (a) SE micrograph of S53P4 fragment (bar = 10 µm); and (b) X-ray dot maps (clockwise from top-left) for Si, K, Ca, and P (tote: the signal of K was amplified to make it more evident, but then, the noise also increased). The arrow (in Fig. 5a) indicates precipitates on the glass surface.


Figure 6. (a) SE micrograph of a S53P4 granule implanted in sheep mandibular bone (bar = 50 µm); and (b) X-ray dot maps (clockwise from top-left) for Na, P, Ca, and Si.


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Figure 7. (a) SE image of an A-W granule implanted in rabbit muscle (bar = 100 µm); (b) BSE image (top left) with X-ray dot maps (clockwise from top-right) for P, Ca, and Si; and (c) X-ray spectra collected from points 1 (solid) and 2 (empty) in Fig. 7a.

Figure 8. (a) SE image of an A-W granule (G) implanted in sheep bone (B) (bar = 100 µm); and (b) the X-ray dot maps (clockwise from top-left) for Mg, P (arrows indicate the P-rich layer), Ca and Si.


Figure 9. IR spectra of Bioglass® granules: (a) after 5 years from preparation and milling; (b) of fresh Bioglass®; and (c) after a 6-month storage.


Discussion with Reviewers

K. de Groot: The overwhelming explanation of calcium phosphate formation on the bioactive glasses and glass ceramics in general accepted concepts seems to be unnecessary.
Authors: The explanation on Ca-P formation is necessary because it stresses the fact that this layer forms also in muscles and not only in bone, and that this formation is different, from one material to another, both for thickness and Ca/P ratio. Furthermore, it is important to note the further Ca,P deposition on the Ca,P-rich layer of S53P4 implanted in muscle.

K. de Groot: There is no proof showing the relation between interfacial reaction of bioglass and its aging, although aging of bioglass may affect its response to biological system.
Authors: We agree that there is no sure proof of the aging-bioactivity correlation, but we first want to advance the hypothesis that aging can be a factor influencing the bioactivity. Further studies must be carried out to demonstrate this.

K. de Groot: The presence of K in Figs. 2(b) and 4(b) requires some explanation. S53P4 does not initially contain K₂O. Can the authors give convincing reasons as to the presence of K in non-degraded center of S53P4 in Fig. 4(b).
Authors: The presence of K can be understood if we consider that in this glass there is an ion exchange with the body fluids. K is probably exchanged with Na. Na is absent.
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K. de Groot: The statement in first paragraph of Discussion "These hydrated layers are weak and easily cracked" lacks evidence.
Authors: The cracks are absent in the unimplanted granules prepared in the same way as the implanted glass (Fig. 1a). The cutting can produce cracks, but only in a "weak" material.

U. Gross: Without histological findings, the presented data cannot be used to compare the changes in implanted particles with the tissue and cellular reactions.

R. Todescan: Since the reaction of bone and "speed up bone regeneration" (in the Abstract) are being taken into consideration in this paper, an histomorphometric analysis of the area of reparative bone for each material should be considered.
Authors: The aim of the work and its originality is to detect the behaviour of the material and present a comparison among them from a materialistic point of view. Their biocompatibility is already well documented (ref. 13, 21 for Bioglass, ref. 2, 9 for S53P4, and ref. 7, 15 for AWG) and widely demonstrated.

U. Gross: How and when did you prepare the granules, before or after ageing?
Authors: All the granules are prepared after melting the glass and used within 6 months, except for Bioglass that was melted, crushed to granules, and stored for 5 years.

U. Gross: How many implantation sites were created and investigated in sheep and rabbits? Why did you use two different time intervals between implantation and explantation in sheep and rabbits? What is the rationale to use sheep and rabbits as species in this investigation? Why did you only use SEM and EDS and not conventional histological techniques in order to investigate the cellular and tissue reactions?
Authors: Every material had two implantation sites in rabbit and two in sheep. Two different time intervals were allowed to elapse since the degradation in muscles is more rapid than in bone. The muscle implantation was selected according to either ASTM or ISO standards and because in the muscular fluids, the ion-exchange is more rapid than in bone. Mandibular sheep bone is used because (1) it has a trabecular and compact tissue, and (2) its growth rate is similar to that of human mandibular bone. Histological findings at low magnification are not provided because in this article we want to stress the different physico-chemical degradations of the materials.

U. Gross: What is the structure and composition of the young and the aged, but not yet implanted, materials in SEM and EDS?
Authors: The chemical composition of the aged Bioglass and new Bioglass is, of course, the same (see Table 1). After a long period, a structural deterioration, at the chemical bond level, occurs; this is detectable by an IR spectroscopy system.

R. Todescan: Since the variable "ageing" of the material is being tested, a non-aged Bioglass should be included in the materials to be tested.
Authors: Since some of the presented results do not properly agree with those previously obtained (21) by one of the authors, the aging of that material was put forward as a hypothesis. Experiments are being carried out to demonstrate clearly that aging is a parameter influencing the bioactivity of the active glasses.

R. Todescan: Can the data in Results be organized and presented in a quantitative form, e.g., i) a table showing the average thickness of the various layers (Ca-P layer, Si-rich layer, etc.) expressed as the ratio of the total surface area of the granule; and/or ii) the profiles obtained with the microprobe (intensity x distance) across the interfaces of the granule, reflecting the concentration of each element. In addition, the results should include data obtained before and after implantation to support statements made in the paper such as "in the original glass the amounts of calcium and phosphorus were lower".
Authors: Measuring the mean thickness is meaningless since the cutting crosses the granules at different levels and the sections obtained present different thicknesses and also different morphologies. Figure 1a shows a glass before implantation along with the corresponding dot maps and elemental spectra.