Microchemical transformation of bioactive glass particles of narrow size range, a 0–24 months study

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Previous studies demonstrated the capacity of bioactive glass particles of narrow size range (300–355 μm, Biogran®) to stimulate bone tissue formation without contact with pre-existing bone tissue. Chemical interactions between the bioactive glass and the surrounding tissue fluids caused the glass transformation. This study quantifies the time-dependent transformation process. Particles were implanted in the jaws of beagle dogs and resected after 1, 2, 3, 6, 12 and 24 months. Microchemical analysis was performed using a scanning electron microscope equipped with an energy dispersive X-ray analysis system. After one month, Na-ions were leached out and the particles transformed into two layers. In the center, a Si-rich gel was found on the outer surface, a Ca- and P-rich shell. After two months, the concentration levels of the outer Ca- and P-rich shell remained. In the center the Si-concentration decreased and the Ca and P concentration increased. After three months, Si disappeared completely from the center of the particle, while the Ca and P concentration increased. At one and two years, the Ca and P concentrations in the transformed particles equalled those of bone tissue, turning the transformed particle into a chemical equivalent of the bone mineral phase.

1. Introduction

Previous studies have shown that bioactive glass particles of narrow size range (300–355 μm) (Biogran®, Orthovita Malvern PA) have the capacity to stimulate the formation of new bone tissue through their osteoconductive and their osteostimulative properties. [1–5]. These properties can be observed histologically and are due to a cascade of reactions and transformations taking place in these glass particles. Eventually, islands of bone tissue form in internally resorbed glass particles.

This reaction of bone stimulation can be summarized as follows [4]. When physiological fluids surround the bioactive glass particles, an ion exchange starts. This leads to the formation of a dual reaction layer: an internal silica-rich gel, surrounded by a calcium–phosphorus-rich (CaP-rich) shell. The particles are fully transformed by virtue of the dimensions of the particles (300–355 μm) and the high turnover of the surrounding tissue fluids. As a result of the chemical changes, dimensional variations occur which provoke cracking of the CaP-rich shell. Phagocytosing cells penetrate through the cracks in the calcium–phosphorus rich shell, enter the central Si-rich part of the particle and resorb the Si-rich gel. In this way the particles become excavated.

Osteoprogenitor cells, present in the surrounding tissues, follow the phagocytosing cells into the center of the particle. They recognize the inner CaP-rich wall as a bone-like substrate, adhere to it and differentiate into osteoblasts. This leads to the formation of islands of new bone in these excavated particles. This newly formed bone is not connected with the surrounding bone tissue. Thus, each particle acts as a nucleus for bone formation, thereby enhancing repair rates of bone defects, beyond those for repair by osteoconduction uniquely.

In this study the chemical changes of the particles are analyzed using scanning electron microscopy and related energy disperse X-ray analysis. The objective is to determine the rate and extent of the chemical transformation of the bioactive glass particles of narrow size range.

2. Materials and methods

Bioactive glass with a nominal composition of 45% SiO_2, 24.5% CaO, 24.5% Na_2O and 6% P_2O_5 (percentage by weight) was cast, crushed and sifted using standardized sieves into particles with a size between 300 and 355 μm. The particles were cleaned ultrasonically with acetone and sterilized with ethylene oxide.

TABLE I Statistical difference between elemental concentrations at the borders of the bioactive glass particles and surrounding bone tissue after respectively 0, 1, 2, 3, 6, 12 and 24 months of implantation

	Silicon			Calcium			Sodium			Phosphorus		
	Borders	Bone	*p*-value	Borders	Bone	*p*-value	Borders	Bone	*p*-value	Borders	Bone	*p*-value
0	44.5 ± 1.28	0.25 ± 0.065	3.27E-14*	14.07 ± 1.02	59.42 ± 0.38	3.75E-15*	32.95 ± 0.99	2.24 ± 0.11	2.22E-13*	8.49 ± 0.9	38.09 ± 5.16	2.43E-14*
1	4.61 ± 0.91	0.25 ± 0.065	3.63E-04*	56.47 ± 0.73	59.42 ± 0.38	0.0033*	2.5 ± 0.09	2.24 ± 0.11	0.13	36.42 ± 0.38	38.09 ± 5.16	0.0046*
2	0.35 ± 0.02	0.25 ± 0.065	0.12	58.42 ± 0.32	59.42 ± 0.38	0.085	2.79 ± 0.33	2.24 ± 0.11	0.095	38.44 ± 0.26	38.09 ± 5.16	0.32
3	0.55 ± 0.15	0.25 ± 0.065	0.088	58.99 ± 0.59	59.42 ± 0.38	0.54	2.86 ± 0.38	2.24 ± 0.11	0.17	37.61 ± 0.21	38.09 ± 5.16	0.29
6	0.72 ± 0.26	0.25 ± 0.065	0.081	59.81 ± 0.76	59.42 ± 0.38	0.60	3.44 ± 0.51	2.24 ± 0.11	0.042	36.08 ± 0.59	38.09 ± 5.16	0.012
12	0.6 ± 0.2	0.25 ± 0.065	0.11	60.2 ± 0.52	59.42 ± 0.38	0.18	2.33 ± 0.08	2.24 ± 0.11	0.59	36.86 ± 0.4	38.09 ± 5.16	0.0097
24	0.81 ± 0.26	0.25 ± 0.065	0.067	58.98 ± 0.64	59.42 ± 0.38	0.51	3.22 ± 0.50	2.24 ± 0.11	0.093	37.00 ± 0.35	38.09 ± 5.16	0.096

*Statistically significant at $p < 0.01$.

Five mature beagle dogs were used for this experiment. The premolars and first molars in the lower jaw were extracted at least three months prior to implantation. In each of these partial edentulous jaws four buccal and four occlusal cavities were surgically prepared with internally cooled, slowly rotating instruments. In addition, two incisors in each lower jaw were extracted immediately before the implantation of the particles.

The dogs were sacrificed at 1, 2, 3, 6, 12 and 24 months after implantation. The resected specimens were immediately fixed in a solution of one part formaldehyde neutralized with 50 g $CaCO_3$/L and two parts of 80% ethanol. The tissue blocks were dehydrated in a graded alcohol series. After embedding in methylmethacrylate, thin serial sections (100–200 μm) were cut on a sawing microtome (Leitz, Wetzlar, Germany). These sections were ground and polished [1]. The polished sections were sputtercoated with a thin layer of carbon. As control, some unreacted glass particles were embedded in methylmethacrylate and prepared in the same way as described above.

Quantification of the chemical changes was obtained by using a scanning electron microscope (SEM) (Philips SEM 515, Eindhoven, The Netherlands), equipped with a microprobe (Tracor northern microtracer series, USA). In the EDAX system only atoms above the atomic number of carbon can be detected. In this study all the components of bioactive glass have an atomic number above carbon, except for oxygen. In this way the relative amount of silicon, calcium, phosphorus and sodium were measured.

From each animal, in this study, three sections were selected at random. In each section, three particles with a comparable size were selected at random. On each particle, an imaginary line was drawn where the diameter was approximately 300 μm. The relative amounts of P, Na, Si and Ca were measured at 40 equidistant points on this line. This resulted in a measurement approximately every 8 μm. In three sections from each time period three different particles were measured. Thus, nine particles were measured per time period and averages were calculated.

In addition, a line of the same length as the cross-section through the particle was drawn partly through bone and partly through fibrous tissue and measured at 40 points.

Elemental dot mapping were also made in order to visualize the chemical changes. Using this technique one image displays the relative distribution of one element non-quantitatively over the entire particle.

In order to determine whether and when the bioactive glass particles became no longer distinguishable from bone tissue, the difference in elemental concentration between the particles and bone was tested. The data were analyzed using a *t*-test for paired data (Table I). The level of significance was set at $p < 0.01$.

3. Results

The mean values of the concentration of silicon, sodium, calcium and phosphorus across the glass granules are displayed in Figs 1–5 for implantation duration of 0, 1, 2, 6 and 12 months respectively.

For the non-implanted particles, high amounts of silicon and sodium were found throughout the whole cross-section, and relatively low amounts of calcium and phosphorus are noticed.

After one month of implantation, the composition of the granule changed completely. In the center of the particle, the relative amount of silicon increased greatly from about 45% to a maximum of 80%. The sodium content diminished to nearly 0%, while the relative amounts of calcium and phosphorus remained unchanged in the center of the particles. A very different result was found at the borders of the particles. In these areas, no sodium was left, while the silicon content decreased to nearly 0%. The relative amounts of calcium and phosphorus increased from about 10% to a maximum of 60% and 38% respectively. In between these two totally different zones, i.e. the center of the particles and the borders, a transitional zone was observed.

After two months of implantation changes occurred in the center of the particles. The relative amount of silicon diminished greatly to an average of ± 20%. The relative amounts of calcium and phosphorus increased to 38% and 30% respectively. At the borders of the particles the result was comparable to the one-month result. After three months of implantation a similar result was found.

The mean values of the relative amounts of the different components after six months of implantation again showed major changes in the center of the particles, while the borders of the particles remained stable. In the center of the particles the relative amounts of calcium and phosphorus slowly increased, while the relative amount of silicon decreased.

After 12 months of implantation no difference could

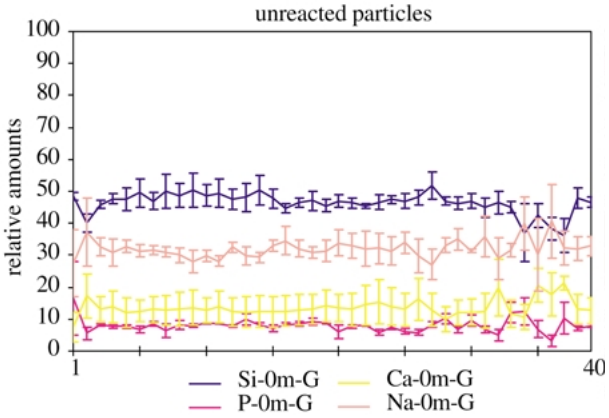

Figure 1 A cross-section of an unreacted bioactive glass particle, regarding the relative presence of Ca, P, Si and Na.

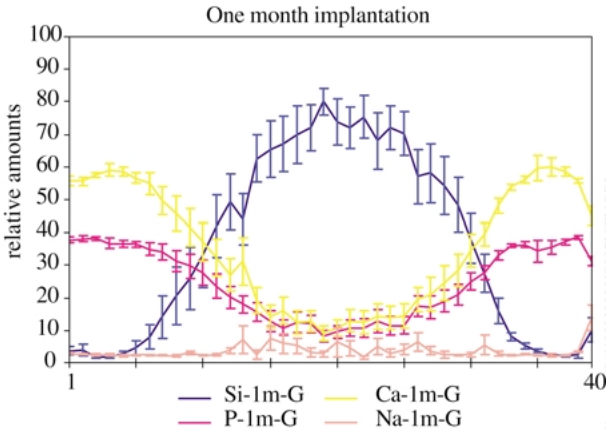

Figure 2 A cross-sectional view on the relative presence of Ca, P, Si and Na throughout the bioactive glass particles after one month of implantation.

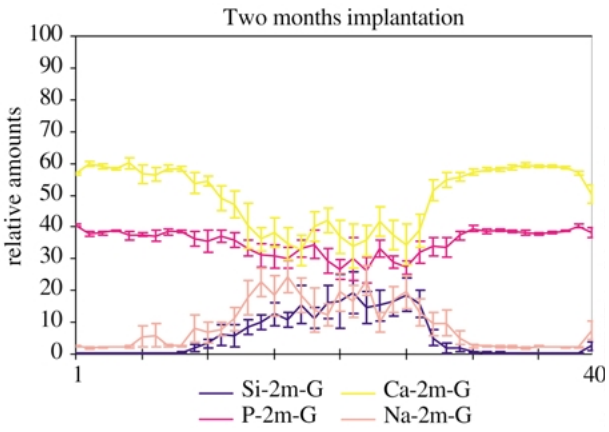

Figure 3 A cross-sectional view on the relative presence of Ca, P, Si and Na throughout the bioactive glass particles after two months of implantation.

be found between the center of the particles and the borders. The relative amounts of silicon and sodium decreased to 0% and 4% respectively. The relative amounts of calcium and phosphorus were 58% and 38% respectively throughout the particle.

After 24 months of implantation a result comparable to the 12-month results was found. The relative amounts of silicon, sodium, calcium and phosphorus remained

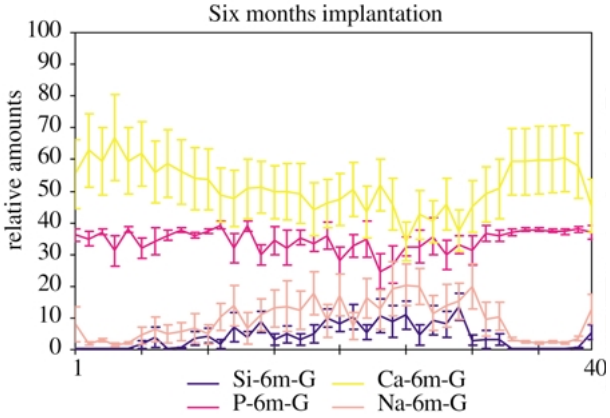

Figure 4 A cross-sectional view on the relative presence of Ca, P, Si and Na throughout the bioactive glass particles after six months of implantation.

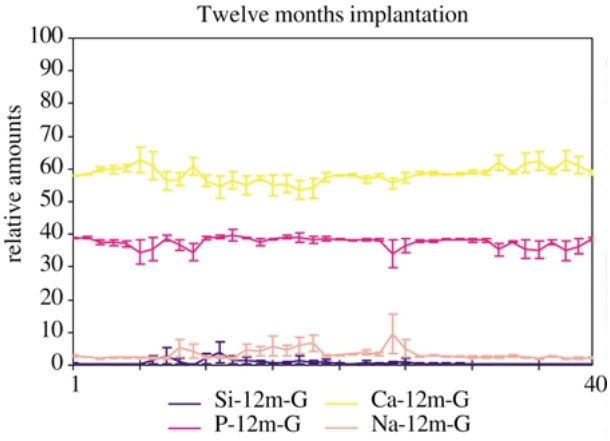

Figure 5 A cross-sectional view on the relative presence of Ca, P, Si and Na throughout the bioactive glass particles after 12 months of implantation.

unchanged, 0%, 4%, 58% and 38%, respectively, throughout the particle.

Bone tissue at the border of the cavity was also measured in order to compare the relative amounts of silicon, calcium, phosphorus and sodium. The results showed the absence of silicon and a very low amount of sodium (nearly 0%). The relative amounts of calcium and phosphorus were 59% and 38%, respectively. Table I shows the statistical comparison between the relative amount of the different components in the border of the bioactive glass and the bone tissue. Even after two years of implantation there is still a statistical difference between the amount of silicon in the borders compared to that in the bone tissue. In contrast, after two (calcium and phosphorus) or one month (sodium) there is no statistical difference in concentration between the borders of the particles and the surrounding bone.

These changes described above can also be observed in the dot-mapping images (Figs 6–13). Whereas these figures show the changes in the whole particle and its surrounding tissue, they do not provide quantification of the relative amounts of the different components. Regardless, the visualization of the distribution of the various elements is useful. As in the SEM-images, a difference in density is observed in the center and at the

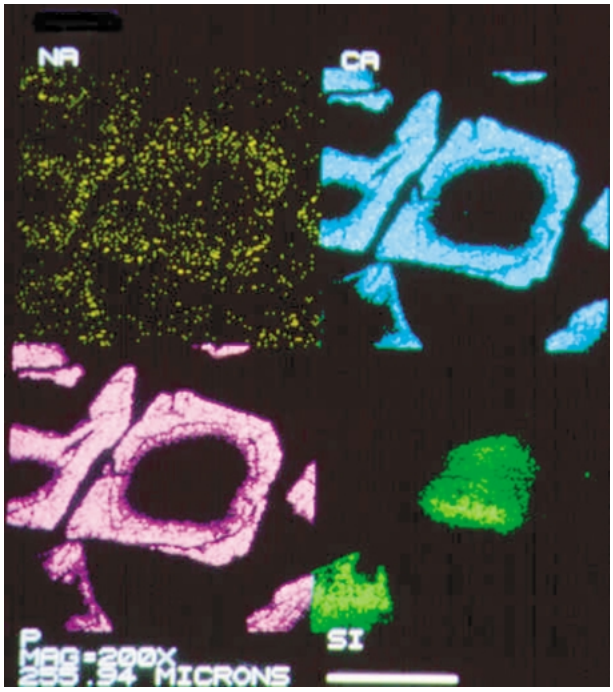

Figure 7 The dot mapping of the particle (see Fig. 6) for the four elements separately, i.e. Na, Si, P and Ca.

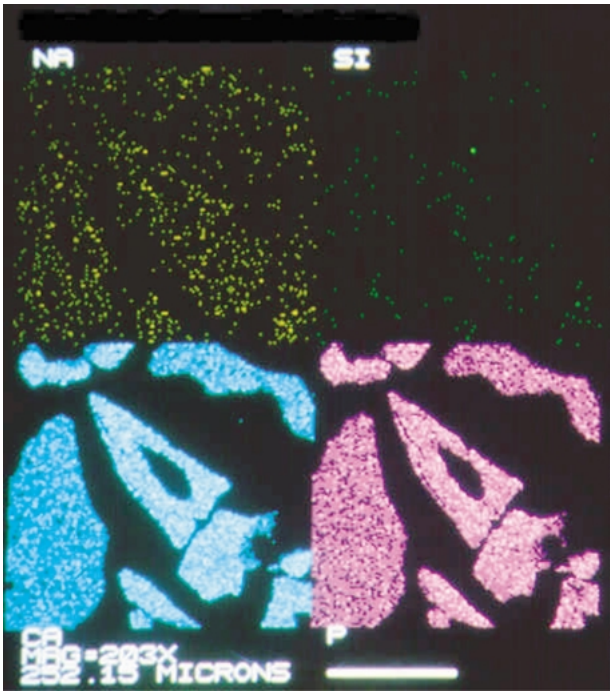

Figure 11 The dot mapping of the particle (see Fig. 10) for the four elements separately, i.e. Na, Si, P and Ca.

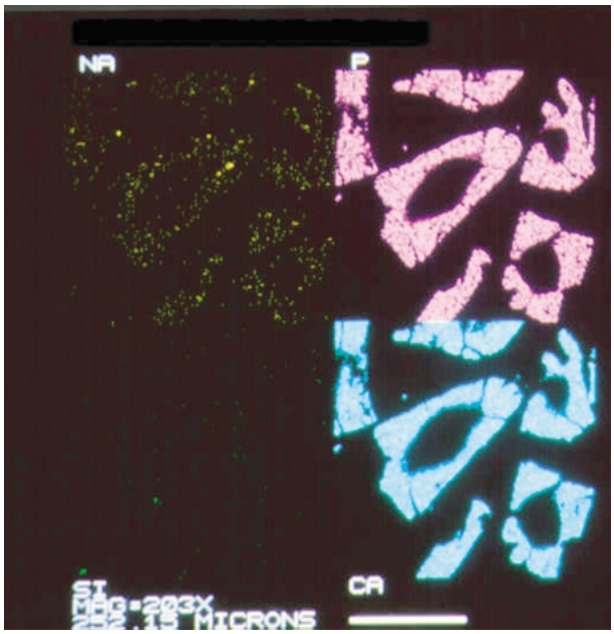

Figure 9 The dot mapping of the particle (see Fig. 8) for the four elements separately, i.e. Na, Si, P and Ca.

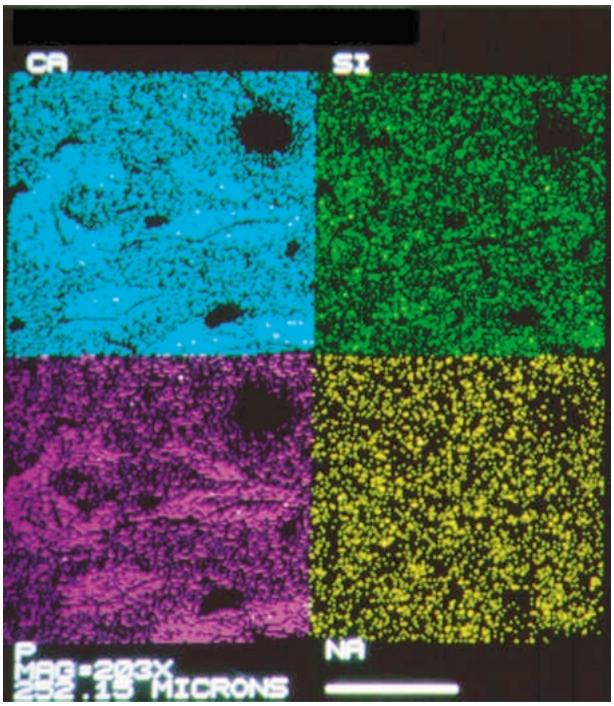

Figure 13 The dot mapping of the particle (see Fig. 11) for the four elements separately, i.e. Na, Si, P and Ca.

borders of the particle after one month of implantation, in contrast to no change in the unreacted particle. In the dot-mapping images, high amounts of silicon are noticed in the center of the particle, and high amounts of calcium and phosphorus at the borders of the particle. There is a difference in the density of the surrounding bone and the borders of the glass granule: several cracks throughout the particles are observed.

After two months and three months of implantation the granule appears to become excavated. A denser appearing granule borders the excavated lumen.

After six months of implantation, bone-formation in the excavated lumen is noticed. The presence of silicon decreased and the presence of phosphorus and calcium increased.

After 12 and 24 months, more granules are surrounded by bone tissue and it becomes more difficult to distinguish the original outline of the particle.

4. Discussion

SEM-images are frequently used to show or reveal glass particles. Resulting dot-mappings are an inviting manner to show in a non-quantitative way how the particles

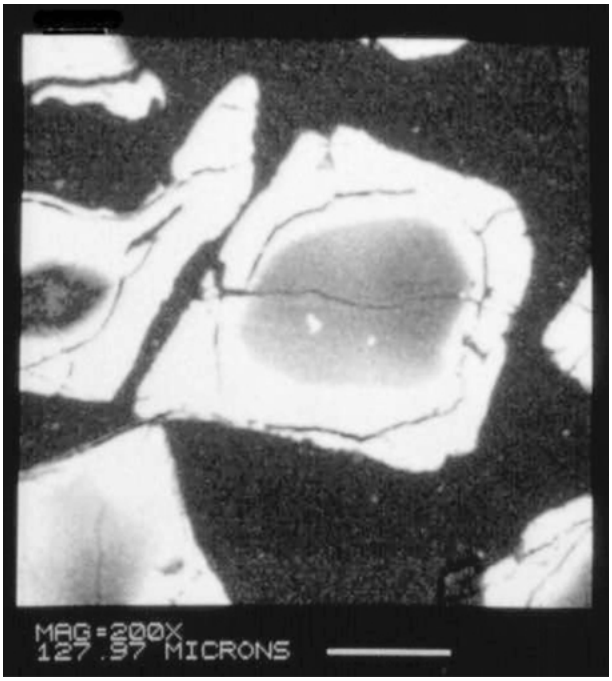

Figure 6 The SEM-image of a bioactive glass particle after one month of implantation.

Figure 10 The SEM-image of a bioactive glass particle after six months of implantation.

Figure 8 The SEM-image of a bioactive glass particle after two months of implantation.

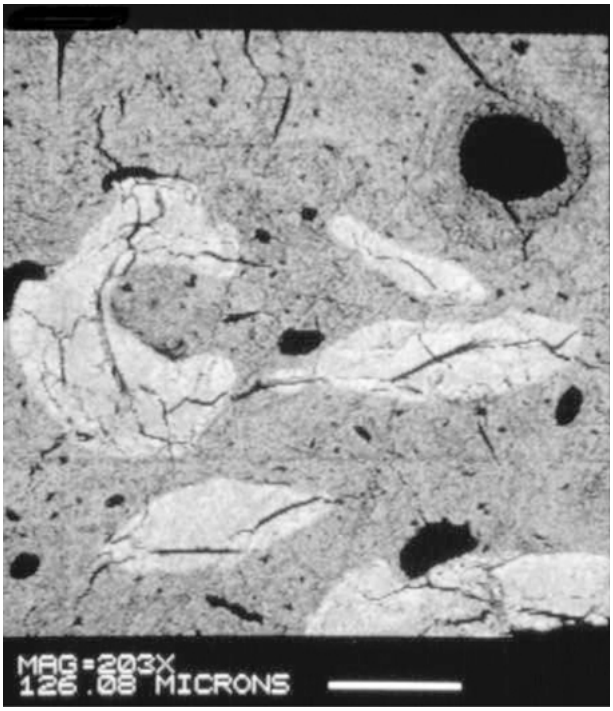

Figure 12 The SEM-image of a bioactive glass particle after 12 months of implantation.

change, as did Furusawa and Mizunuma [9] for implantation of bioactive glass in humans up to seven months [6]; and Gatti and Zaff [7] in sheep after two months of implantation [7]. Thus, the measuring methods have shown their usefulness. Line-scans are not often used [4], but in contrast to point-analysis, line-scans give a more accurate image and a better overview.

The results given by the line-scans show how the composition of the particle changes throughout time, i.e. from 0 up to 24 months. These results are in agreement with the results of Gatti and Zaffe [7], who measured the relative presence of the different components in a few points of the particles after two months of implantation.

The measuring technique has its own limitations. The X-ray photons detected for the elements of interest are used to calculate their concentrations in the samples. However, not only are the elements of the sample itself measured, elements emit X-rays due to contamination of the sample and X-ray scattering. These ‘‘errors’’ are defined as background scattered energy [8]. The EDAX system calculates the relative amount of the peak

registrations with a correction factor to compensate for these ''errors''.

As mentioned previously, in the unreacted particle measurements, a slightly irregular pattern near the borders of the particles was obtained. This is probably due to a chemical reaction of the particle with the embedding material, i.e. methylmethacrylate. Due to the difficulty of determining the exact border of the particles, an irregular image on the border of the particle was observed in those particles that were implanted. The spatial resolution of the EDAX instrument is limited by the sphere in which the X-rays are produced (1 μm diameter) [4].

After two months of implantation the dot mapping shows an excavation of the particles. The line-scan shows an irregular image of the different components in the inner part of the particles. Schepers *et al.* [1, 4] observed this phenomenon histologically. The irregular image is caused by the invasion of the excavated lumen by fibrous tissue. The three months sections produced an analogous image. After six months of implantation, the dot mapping shows bone-formation in the protective pouches, which were formed as a result of excavation of the particle. The line-scans show an increase of the relative amounts of Ca and P in the excavated lumen, starting from the inner wall of the excavated particles. Twelve to 24 months after implantation, bone-growth has continued and filled up the internal lumen of the particles.

Most of the particles are surrounded by bone after 12–24 months of implantation. The relative amounts of the different components measured in bone or in the remainder of the particles are similar. However, bone and the glass particles are still distinguishable. The line-scans on the contrary show no difference between the relative amounts of the different components of bone compared to the transformed glass particle. So it cannot be stated that the particles have become bone tissue, because a distinction can still be made between bone tissue and glass particles even after two years of implantation. This difference can be due to the specific microstructure of bone that is not found in the glass particles.

Several authors have stated that bioactive glasses transform after implantation [9–14] and thanks to this study, we have quantified the changes that occur.

Since glass particles change into an equivalent of bone tissue, the question raises whether placement of a titanium implant would be possible in a site, previously implanted with bioactive glass particles. Previous studies showed the relative ease [14, 15] of the installation of implants in such an area in contrast to other osteoconductive or -inductive materials.

Schepers *et al.* [15] showed evidence that the remodeling of the glass particles is influenced by the remodeling activity of the surrounding bone. The chemical reactions of the particles will go faster in a site with high remodeling activity than in a site with lower activity, as in this study. Near an implant surface where high remodeling activity takes place, most of the particles where reduced in diameter and showed a widened entrance to the internal lumen. At a distance, where limited remodeling activity took place, the granules where larger and less reduced.

5. Conclusion

Several authors have stated that bioactive glasses transform after implantation. Even if the glass particles come in contact with body simulated fluids in *in vitro* experiments this transformation occurs, to various extents. Results from this study show that this transformation leads to the formation of a bone-like substrate, which has the same chemical composition, but not the same structure. This results in a different density, so the glass particles can still be distinguished microscopically and electron-microscopically.

References

1. E. J. G. SCHEPERS, M. DECLERCQ, P. DUCHEYNE and R. J. KEMPENEERS, *J. Oral Rehab.* **18** (1991) 439–452.
2. P. DUCHEYNE, A. EL-GHANNAM and I. SHAPIRO, *J. Cell. Biochem.* **56** (1994) 162–167.
3. P. DUCHEYNE, P. BIANCO, S. RADIN and E. SCHEPERS, in ''Bone Bonding Biomaterials'' (Kokubo & Van Blitterswijk, Leidorp, 1992) 1–12.
4. E. SCHEPERS and P. DUCHEYNE, *J. Oral Rehab* **24** (1997) 171–181.
5. A. J. GARCIA and P. J. DUCHEYNE, *J. Biomed. Mater. Res.* **28** (1994) 947–960.
6. T. FURASAWA and K. MIZUNUMA, *Implant. Dent.* **6** (1997) 93–101.
7. A. M. GATTI and D. ZAFFE, *Biomaterials* **12** (1991) 497–504.
8. G. DACULSI and R. Z. LEGEROS, *Scan. Micros.* **4** (1990) 309–314.
9. T. FURUSAWA and K. MIZUNUMA, *Implant. Dent.* **6** (1997) 93–101.
10. S. RADIN, P. DUCHEYNE, S. FALAIZE and A. HAMMOND, in ''Bioceramics'' (Sedel & Rey, Cambridge, 1997) 45–48.
11. I. M. O. KANGASNIEMI, E. VEDEL, J. DE BLICK-HOGERWORST, A. U. YLI-URPO and K. DE GROOT, *J. Biomed. Mater. Res.* **27** (1993) 1225–1233.
12. A. EL-GHANNAM, P. DUCHEYNE and I. M. SHAPIRO, *Biomaterials* **18** (1997) 295–303.
13. T. KOKUBO, *J. Non-Cryst. Sol.* **120** (1990) 138–151.
14. O. P.FILHO, G. P. LATORRE and L. L. HENCH, *J. Biomed. Mater. Res.* **30** (1996) 509–514.
15. E. SCHEPERS, L. BARBIER and P. DUCHEYNE, *Int. J. Oral. Maxillofac. Implants.* **13** (1998) 655–665
16. L. BARBIER, J. VANDER SLOTEN, G. KRZESINSNSKI, E. SCHEPERS and G. VAN DER PERRE, *J. Oral Rehab.* **25** (1998) 847–858.

Received 28 September
and accepted 1 November 2000