Granule size and composition of bioactive glasses affect osteoconduction in rabbit

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Bioactive glass granules of three different compositions, regarding particularly Si- and Al-content (S53P4, S59.7P2.5, S52P3) and of two different granule sizes (200–250 μm and 630–800 μm) were implanted for 4 and 8 weeks in the distal part of rabbit femur. The effect of glass composition and granule size on bone formation was studied. The results were evaluated using histology, computerized histomorphometry, scanning electron microscopy and energy dispersive X-ray analysis, and used for mathematical description of bone formation.

The results showed that both the composition of the glass and the granule size of the granules, have influence on bone growth from the surrounding tissue. Glass S53P4, which from previous observations is known to be an effective bioactive glass and widely used in the Biomaterial Project of Turku, Finland, showed bone bonding and increasing bone growth between the granules. Glass S59.7P2.5 which due to its high Si-content should be inert, showed bone bonding. At 4 weeks the bone growth was significantly more abundant in bone defects filled with large granules (630–800 μm) than in defects filled with small granules (200–250 μm). Glass S52P3 with an alumina content of 3 wt %, showed good bone conduction, possibly even bone bonding for granules of 630-800 μm size. Granules of 200–250 μm with a high alumina content at the surface of the reaction layer, showed hardly any bone contact at all.

This data, therefore, gives new information concerning bone bonding and osteoconduction of bioactive glasses with a high silica or alumina content.

1. Introduction

Bioactive glasses are bone graft substitutes, which can form a chemical bond with living tissue [1, 2]. In contact with body fluids, the surface of a bioactive glass is transformed to a Si-rich layer. The appearance of the bond between the implant and the tissue is related to the development of a hydroxy carbonate apatite reaction layer on the surface of the implanted material [3–8].

The bioactivity of the glass has shown to be composition dependent. In a SiO_2-Na_2O-CaO system with a constant P_2O_5 content of 6 wt %, glasses with a silica content of 60 mol % or more have been reported not to be bioactive [2].

Many bioactive glasses show a high solubility, which may reduce their long-term reliability. The solubility of a glass can be controlled by addition of Al_2O_3. The addition of alumina of more than 1.5 wt % will, however, disturb the mineralization of the osteoid, as well as affect the surface reactions of the glass inhibiting Ca/P-formation [9].

In addition to their bone bonding ability bioactive glasses can conduct bone growth along their surfaces and their reactivity can be controlled by choice of glass composition. The reactivity of the glass has also been thought to depend on the surface area to solution volume (SA/V). It is, however, not known if the SA/V ratio of a bioactive glass affects bone growth.

The aim of this study was to quantitatively evaluate bone formation in cavities filled with bioactive glasses of different composition and granule size.

2. Materials and methods

2.1. Glass preparation

Three different glasses were chosen according to suspected differences in osteoconductive behavior (Table I). In the preparation of the glasses, the raw materials were SiO_2, Na_2CO_3, $CaCO_3$, $CaHPO_4.2H_2O$, H_3BO_3 and Al_2O_3. The glasses were melted in a platinum crucible at 1360 °C for 2.5 h. The melt was annealed, crushed and sieved into two different sizes: 200–250 μm and 630–800 μm.

2.2. Animals and surgery

Twenty nine-week-old rabbits whose weight ranged from 1.4 to 2.0 kg were used. The operations were performed

TABLE I Glass composition in wt %

Glass	Na_2O	CaO	P_2O_5	B_2O_3	Al_2O_3	SiO_2
S53P4	23.0	20.0	4.0	0.0	0.0	53.0
S59.7P2.5	25.5	11.0	2.5	1.3	0.0	59.7
S52P3	18.0	24.0	3.0	0.0	3.0	52.0

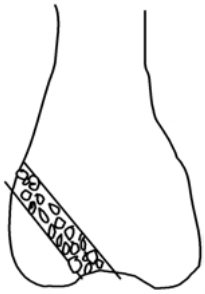


Figure 1 Schematic presentation of operation technique and implantation of graft material in the distal part of the rabbit femur.

with the animals under general anesthesia using intramuscular fentanylfluanisone. A channel with a diameter of 3.2 mm was surgically created in the distal femur using an Astra Meditec 6124SPS drill, starting inta-articularly beside the cartilage layer at the fovea intercondylaris and ending at the cortex. The length of the channel was about 1.5 cm (Fig. 1).

Five different combinations of glass compositions and sizes of glass granules were used (Table II). For each combination of composition of glass and granule size and time (4 and 8 weeks) four parallel experiments were performed. The combinations were chosen by means of a computer search method for minimum correlated stochastic design of the experiment [10]. Using this Plackett and Burman method the sufficient number of experimental animals is systematically optimized [11]. The sterilized glass granules were implanted in the bone channel. The animals were given one single dose of procain penicillin to prevent infections and the analgesic buphrenorphin for three days postoperatively.

2.3. Preparation of specimen

Ten animals were killed at four and at eight weeks respectively. The bones containing the glass granules were fixed in buffered formaldehyde. The samples were dehydrated in increasing concentrations of ethanol and methylmethacrylate for two months and finally embedded in methylmethcrylate (Technovit, Kulzer GmbH, Wehrheim, Germany). Using a cutting-and-grinding technique [12], 20 μm thick undecalcified sections were prepared. The specimens were stained with toluidine blue and van Gieson method and the remaining blocks were studied using scanning electron microscopy (SEM) and energy dispersive X-ray analysis (EDXA). The preparation of the glass and the specimens were performed according to Good Laboratory Practice (GLP) standard.

TABLE II Combination of glass and granule size

Experiment	Glass	Granule size (μm)
1	S53P4	200–250
2	S59.7P2.5	200–250
3	S59.7P2.5	630–800
4	S52P3	630–800
5	S52P3	200–250 + 630–800 (1 : 1)

2.4. Histomorphometry

The amount of bone was measured using a Kontroc Electronic/VIDAS 2.1C computer system for histomorphometric measurements. For every specimen three different measurements from a standardized area of the channel were performed. As every combination of graft material was implanted four times, the bone formation in percentage is the result of twelve measurements for each group.

2.5. Statistics

The histomorphometric results, were evaluated by a statistician with two-way variance, student-*t*-test and Mann-Whitney *U*-test.

3. Results

3.1. Histological examination

Inspection by light microscopy revealed no inflammatory cell reactions. The bone grew predominantly centripetally from the sides of the channel to the inside. Fibrous tissue encapsulation was often found around the small granules of the alumina containing glass. No difference in bone growth at the proximal or at the distal side of the epiphyseal line was observable.

3.2. Histomorphometry

The histomorphometrical results of bone growth are shown in Table III.

Glass S53P4 showed extensive bone growth between the granules (Fig. 2), which increased with observation time. Compared to S53P4, the alumina containing glass S52P3 and the silica containing glass S59.7P2.5 at 8 weeks, showed significantly less bone formation between the granules ($p < 0.001$). The bone formation was also depending on granule size. A mixture of S52P3 (200–250 μm/630–800 μm) showed less bone formation than S52P3 (630–800 μm) alone ($p < 0.039$ at 4 weeks and $p < 0.050$ at 8 weeks) (Fig. 3(a), (b)).

TABLE III Histomorphometrical results of bone growth in rabbit femur. Mixture = 200–250 + 630–800 μm 1 : 1

Glass	Granule size (μm)	Time (weeks)	% bone + SD
S53P4	200–250	4	40.1 ± 6.4
S53P4	200–250	8	61.8 ± 7.9
S59.7P2.5	200–250	4	39.5 ± 6.4
S59.7P2.5	200–250	8	48.6 ± 7.1
S59.7P2.5	630–800	4	47.8 ± 7.0
S59.7P2.5	630–800	8	49.0 ± 7.0
S52P3	630–800	4	35.5 ± 6.1
S52P3	630–800	8	48.3 ± 7.3
S52P3	mixture	4	28.5 ± 5.5
S52P3	mixture	8	35.3 ± 5.9

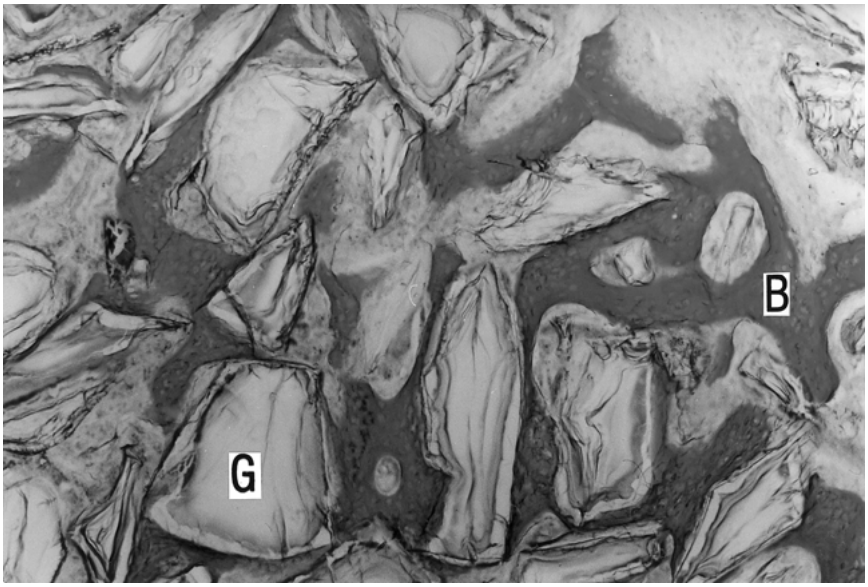


Figure 2 Glass S53P4 of 200–250 μm granule size after 4 weeks of implantation. G = glass, B = bone.

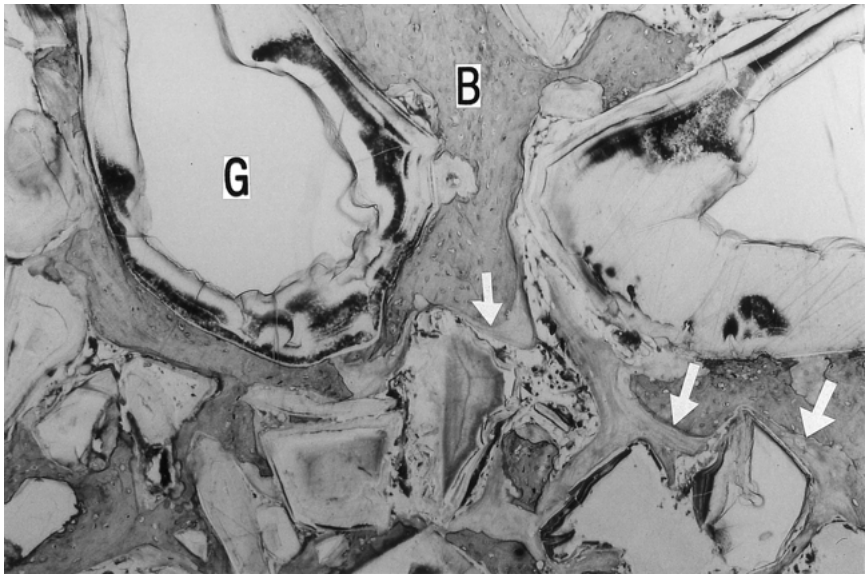


Figure 3a Glass S52P3 200–250/630–800 μm 1 : 1 mixture after 8 weeks of implantation. Note the fibrous tissue encapsulation around the small granules (arrows) and abundant bone formation around the large granules. G = glass, B = bone. M × 10.

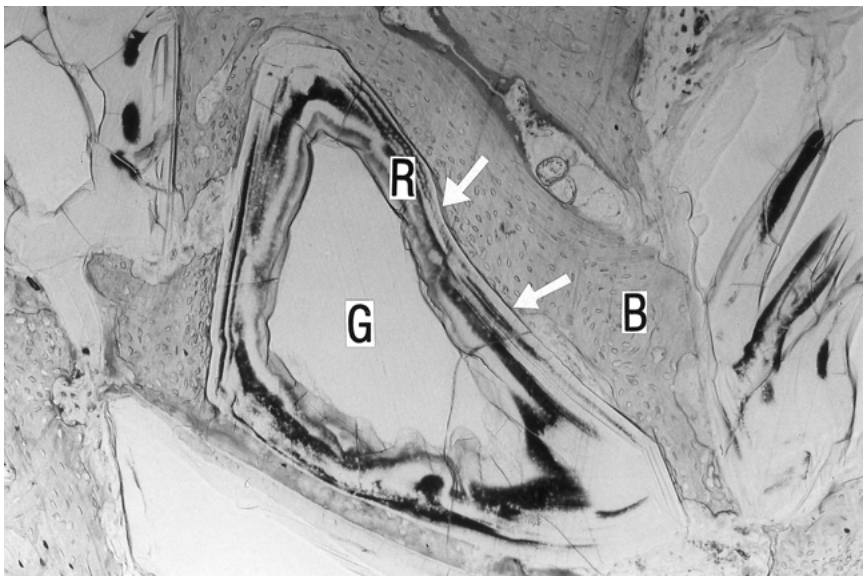


Figure 3b Glass S52P3 630–800 μm granule size, after 8 weeks of implantation. Note the reaction layer of the alumina containing (3 wt %) glass and the good bone contact (arrows). G = glass, B = bone, R = reaction layer. M × 10.

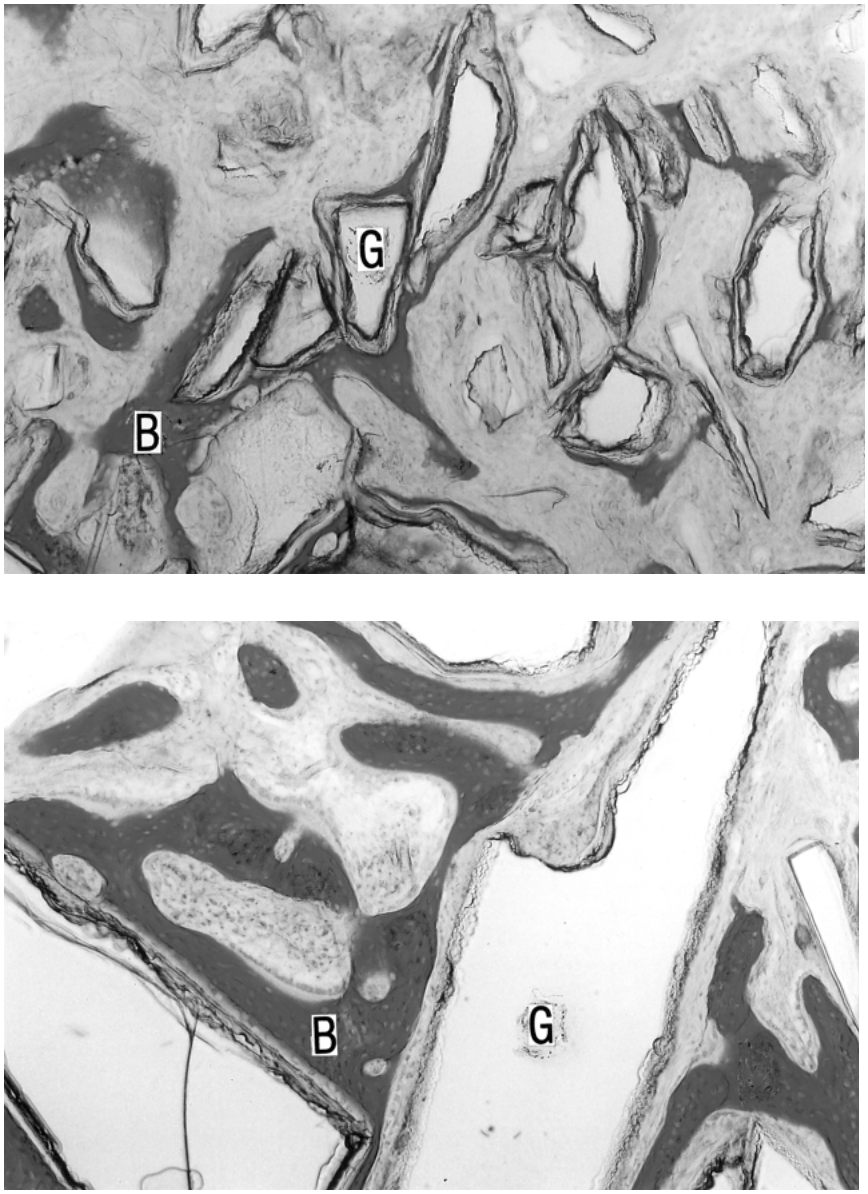


Figure 4 Glass S59.7P2.5 of 200–250 μm size (a) and 630–800 μm size (b) after 4 weeks of implantation. Note the difference in the amount of bone formation around the granules. G = glass, B = bone. M × 10.

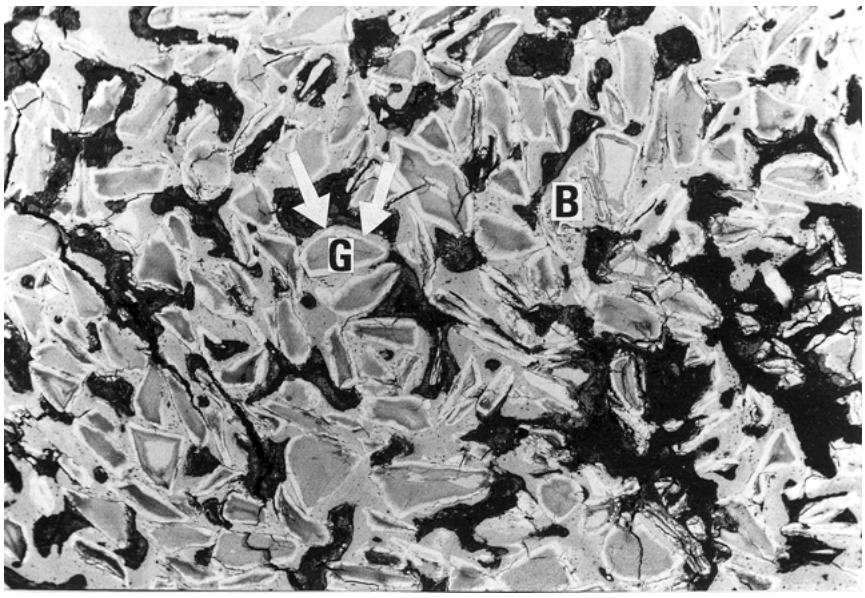
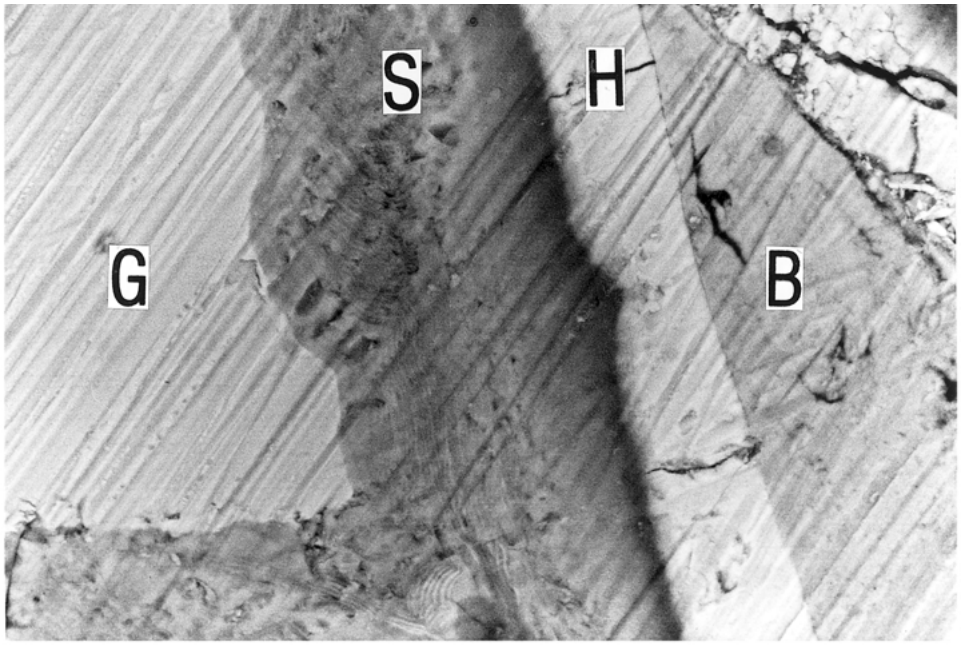
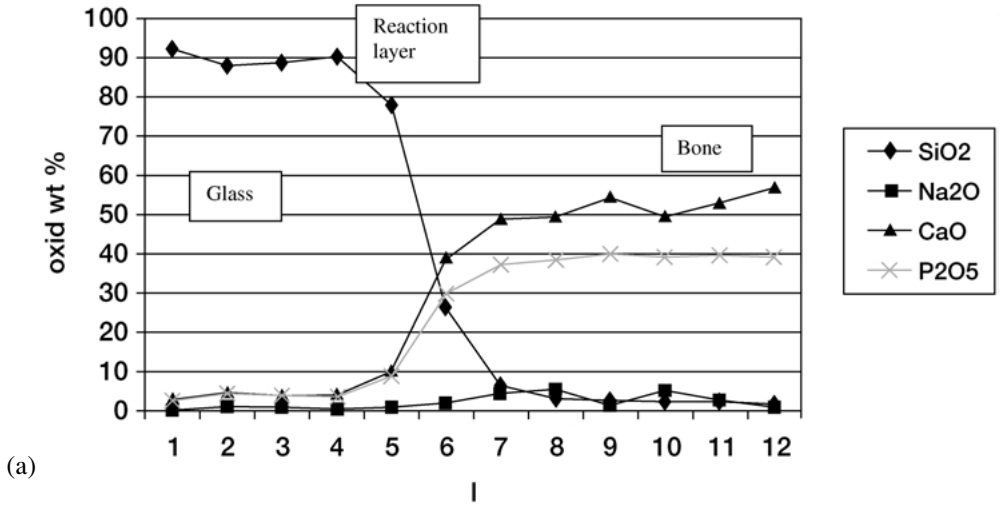


Figure 5 SEM picture of glass S53P4 after 8 weeks of implantation. Note the typical hydroxyapatite phosphate reaction zone as white layer around the granules (arrows), and the extensive bone formation around them. G = glass, B = bone. (1 cm = 0.3 mm).





(a)

Figure 6 SEM images of glass (a) S53P4, (b) S52P3 and (c) S59.7P2.5 with corresponding EDXA profiles. (a) and (c) show bone bonding, (b) shows bone contact, possibly even bone bonding. G = glass, B = bone, S = silica layer, H = hydroxyapatite layer, R = reaction zone. (1 cm = 10.8 μm (a), 14.3 μm (b) and 12.5 μm (c)).

For glass S59.7P2.5 the differences in bone formation depending on glanule size was also observable but only in the beginning of the implantation period. At 4 weeks the bone formation was significantly smaller ($p < 0.006$) for granules of 200–250 μm than for granules of 630–800 μm size (Fig. 4(a), (b)). At 8 weeks no difference in bone formation was observable.

3.3. SEM/EDXA-analysis

All granules of S53P4 had developed the characteristic Ca,P-rich layer of approximately 15 μm and showed bone bonding (Figs. 5, 6(a)).

Granules of S52P3 of 630–800 μm size showed good bone contact, possibly even bone bonding both alone and in the mixture (Fig. 6(b)), whereas granules of 200–250 μm hardly showed any bone contact at all. For granules of 630–800 μm with bone contact EDX-analysis showed 4.5–6.5 wt % Al_2O_3, and Ca,P accumulation within the reaction layer. Granules without bone contact showed more than 10 wt % Al_2O_3 at the surface, with no observable Ca,P-rich layer.

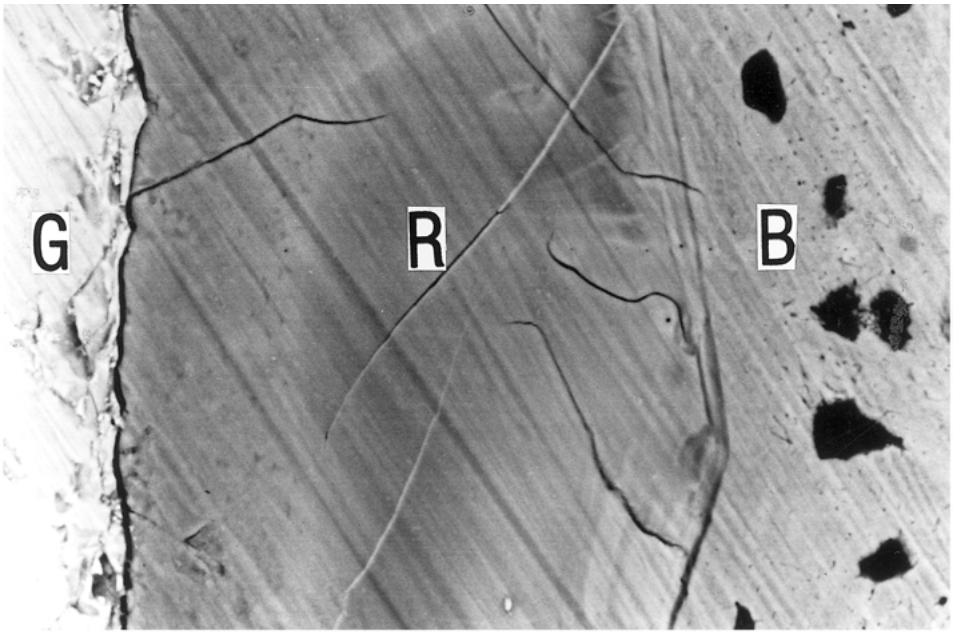
Bone bonding for glass S59.7P2.5 for both granule sizes was observable. Granules to which bone had bonded showed a Ca,P-rich surface layer of 10 to 15 μm (Fig. 6(c)).

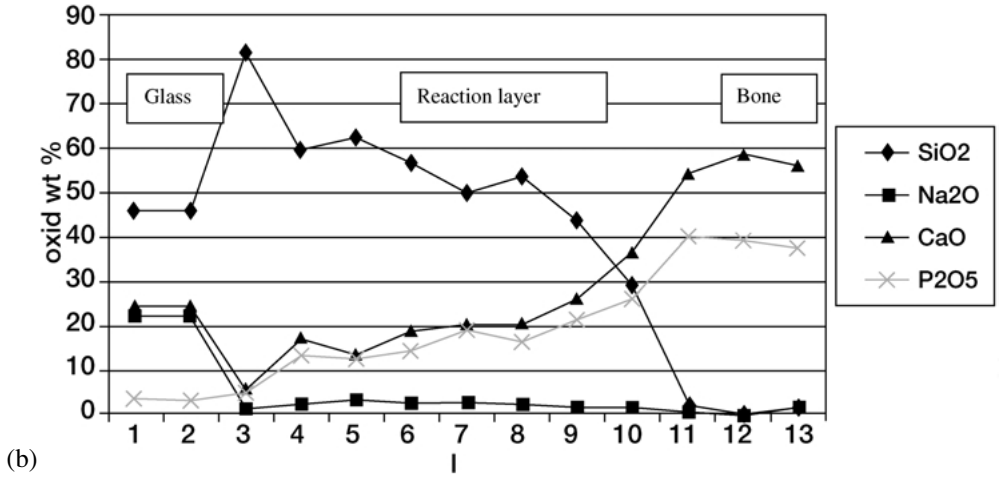
3.4. Phenomenological model

The experimental model was designed for phenomenological description of bone formation [10, 11]. The best fit was obtained by a backward elimination procedure and was found to be:

$$y(\%) = 40.72 - 6.65(p\mathrm{B_2O_3} - 0.52) - 6.71(p\mathrm{Al_2O_3} - 1.20) + 19.32(d - 0.47) + 2.98(t - 5.33) \quad (1)$$

y is the amount of formed bone in % for the standard area at $\times 10$ magnification, p is the composition of oxide in





(b)

Figure 6 (Continued)

wt %, d is the mean diameter in mm of granule size and t is time in weeks. The correlation coefficient, R^2, is equal to 82.98%.

Time t is a linear fit between two points. Extrapolation outside the time range (4–8 weeks) may therefore, be erroneous. The tested variables were: amount of oxides $p(SiO_2,\ Na_2O,\ CaO,\ P_2O_5,\ B_2O_3,\ Al_2O_3)$, d, t, and staining method. Only pB_2O_3, pAl_2O_3, d and t were found to have significant influence on bone formation. The validity range of Equation 1 is given in Tables I and II.

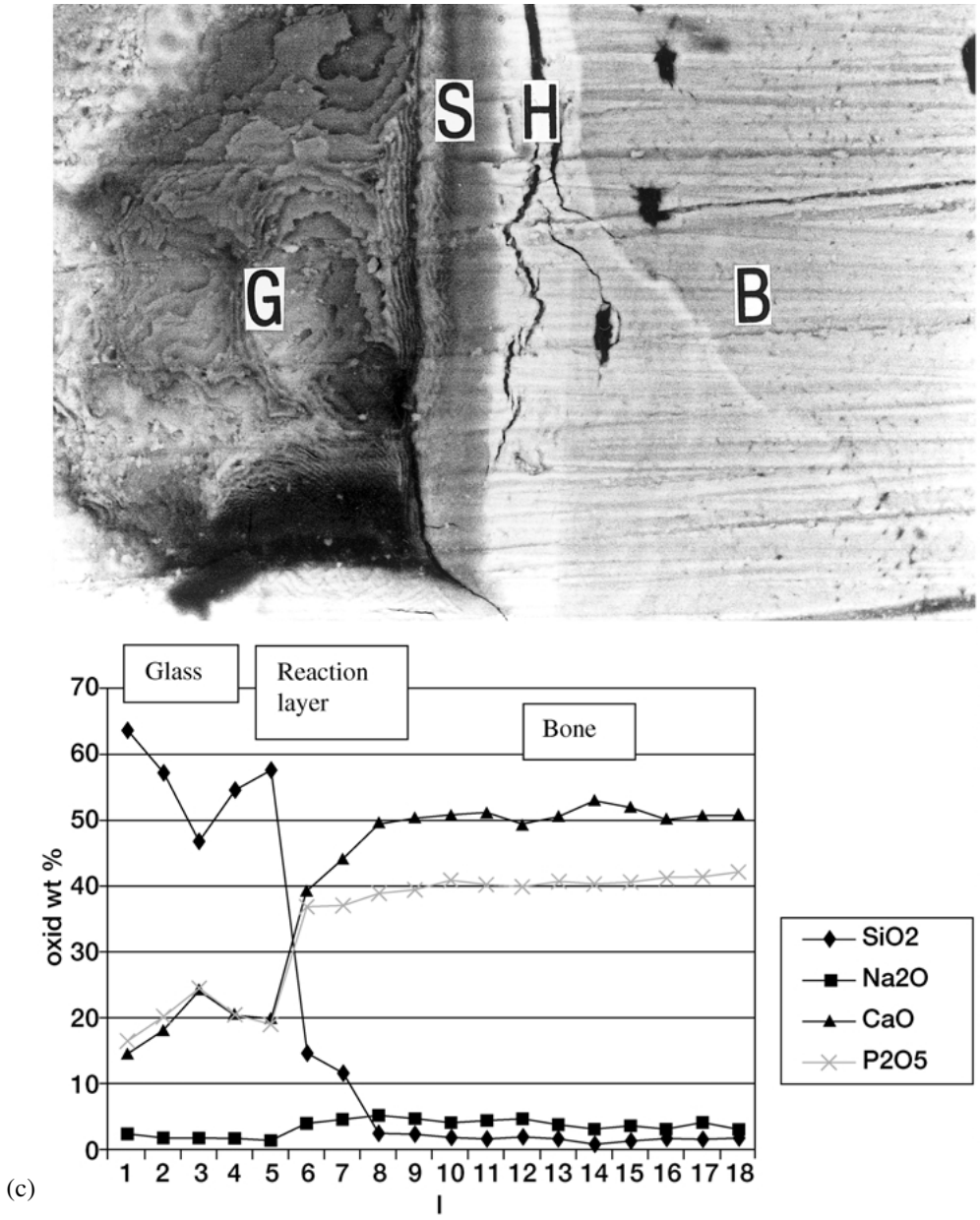
4. Discussion

Our study shows that both the composition of the implanted glass and the size of the granules, will have influence on the subsequent growth of bone from the surrounding tissues.

Glass S53P4, which from previous studies is known to be a highly bioactive glass and served as a control, behaved as expected with a large network of growing bone between the granules [13, 14].

Interestingly the amount of growing bone for glass S59.7P2.5, which according to the literature should be an inactive glass, at 4 weeks was about the same as for glass S53P4. The difference between bone formation for glass S59.7P2.5 at 4 weeks for the two particle sizes (200–250 μm and 630–800 μm) was significant, suggesting that the small granules have an adverse effect on bone growth especially in the early phase of the bone formation process. The smallest amount of growing bone between granules was measured for the granular mixture fraction of glass S52P3. This may depend on several factors. The measurable area between the particles is smaller compared to other areas, which means that it is impossible for bone to grow to the same extent, as the interconnecting macropores may not provide necessary spatial arrangements for capillar and bone in growth. The mixture also contains small granules, which can have a negative effect on growing bone.

In a comparative study for several bioceramic granules of different size and composition the bone growth varied with the kind of size and ceramic used [15]. Similar results have been observed using Bioglass® suggesting



(c)

Figure 6 (Continued)

that the bone formation between large granules is larger than between small granules [16]. Why would differences in granule size have influence on bone formation Gatti *et al*. [17] have implanted granules of glass S53P4 of three different granule sizes (180–250, 300–500, 630–800 μm) in muscle pockets in rabbits, to evaluate the influence of granule size on glass transformation and surface reactions. By using a gold coating method, two sides with different permeability were obtained for the granule. This revealed a two directional diffusion mechanism; one from the glass to the surface and another from the solution to the glass. The chemical transformation and degradation of the glasses were, however, equal regardless of granule size, as the thickness of the reaction layer was the same. In small granules where the reaction layer is about half of the granule size, there will, however, be no Si barrier to depress ion diffusion, and the erosion of the glass may continue leading to glass dissolution [18, 19].

In a model for clinical sinus obliteration with bioactive glass (S53P4), differences between Si and P dissolution from the glass in phosphate buffered saline solution, depending on granule size have been observable. The loss of both ions increased more significantly with small granules (630–800 μm) than with large granules (800–1000 μm). Corresponding decrease was also observable in density measurements using region of interest technique by computed tomography [20]. The surface area to solution volume ratio (SA/V) will, therefore, have influence on the ion exchange in tissue solutions [21] and pH, leading to differences in biological tissue responses i.e. bone formation.

Alumina has a retarding effect on the glass dissolution process and the solubility of the glass can, therefore, be controlled by Al_2O_3 addition. Alumina may also stabilize the glass structure leading to complete inhibition of Ca,P formation at the glass surface, with subsequent loss of bioactivity. Andersson *et al*. have shown, that the alumina containing bulk glass, S52P3, implanted in rabbit tibia, undergoes extensive reactions with formation of thick silica-rich layers, but with no calcium-phosphate formation at the surface and no bone contact

[22]. We have, however, observed granules with calcium-phosphate accumulation in the reaction layer with subsequent good bone contact, indicating that results for bulk glasses are not always equivalent with granules. Glass S52P3, compared to S53P4 and S59.7P2.5 at four weeks, however, showed least bone growth between the granules. It is known from previous observations that a concentration of more than 1.5 wt % of alumina disturbs the mineralization of the osteoid [23–25]. The high alumina value of 10 wt % measured by EDXA at the surface of small granules of S52P3 is apparently an important factor explaining the poor bone contact. This is also shown in the phenomenological description, where a high concentration of alumina leads to a smaller amount of bone growth, as alumina is a significant variable in the equation.

The equation can also be used for estimation of the effect of alumina addition to various glasses and the expected bone formation. In a phenomenological description for bone formation Andersson *et al*. [26] have shown, that a glass with a higher SiO_2-content needs a lesser alumina addition in reducing the solubility of the glass. Our model shows that when the alumina content in the glass is increased, the glass granule size has to be larger, if the same amount of bone formation around the granules is desirable.

Bone healing in a rabbit model is known to have a high bone formation rate. Differences in bone formation in such good healing circumstances gives the thought, that it seems like there would be an optimum size between bulk and small granules where different glasses show most bone growth and osteoconduction. This is most likely related to differences in the SA/V ratio, which emphasizes the importance of studying the effect of this phenomenon.

5. Conclusions

The main findings of this study is, that the composition and the size of the granule of bioactive glasses have influence on bone formation and bone conduction.

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