Primary bone-derived cell colonization of unconditioned and pre-conditioned Bioglass 45S5 surfaces *in vitro*

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Samples of Bioglass 45S5, a bioactive glass that reacts chemically on immersion in an aqueous environment and following implantation can bond with bone, were immersed in culture medium with foetal calf serum as a source of proteins for periods of 1–11 days. Energy dispersive spectroscopy (EDS) revealed that over 11 days immersion, the Bioglass surface was rich in Si relative to Ca and P and scanning electron microscopy (SEM) showed that the Bioglass developed surface reaction layers. Samples conditioned in culture medium and previously untreated samples were used as substrates for primary osteoblast or periosteal cell cultures, to allow comparison of the effects of different Bioglass surface development, on subsequent cell attachment. SEM examination revealed that pre-conditioned Bioglass was a more suitable substrate for osteoblast colonization than previously undeveloped Bioglass, whereas periosteal cells colonized all samples equally well and more rapidly than osteoblasts.

Introduction

One of the general problems in bone and joint replacement surgery is fixation of the bone substitute material to bone. Consequently for the majority of implants, failure originates at the interface between the biomaterial and the host bone [1].

In an attempt to achieve more stable fixation of bone implants, bioactive glasses and glass ceramics have been developed with the introduction of Bioglass in the early 1970s [2]. Bioactivity has been defined as ''the characteristic of an implant material that allows it to form a bond with living tissues'' [3]. Bioactive prostheses react chemically on implantation into the body, in a way that is compatible with the repair processes of the tissues and a fibrous capsule is prevented from forming by the adhesion of newly synthesized bone [2].

The majority of studies undertaken to investigate surface development of Bioglass *in vitro* attempting to elucidate the mechanism responsible for the bone bonding behavior of this material, have utilized simulated body fluid (SBF) or tris-buffer solutions with ion concentrations comparable with that of human blood plasma [4, 6, 20]. Techniques such as X-ray diffraction [4], Fourier transform infrared reflection spectroscopy [4, 6, 20] and Auger electron spectroscopy [6] have been employed to examine the surface structural changes of CaO, SiO_2-based glasses after immersion in SBF or Tris-buffer for various times. These studies revealed that glasses which developed a surface apatite layer after soaking in SBF did so at different rates according to their compositions, although the process of surface structural change was essentially the same in each case. Initially a silica hydrogel formed on the glass surface followed by amorphous calcium phosphate on top of the silica and later an apatite layer that grew with increasing immersion time [4]. The importance of the hydrated silica layer for inducing apatite formation was confirmed by demonstrating formation of apatite on the surface of a pure hydrated silica gel in SBF when the pH was increased from 7.2 to 7.4. It was suggested that silanol groups abundant on the surface of the silica gel were responsible for apatite nucleation [5]. It has also been proposed, after reacting Bioglass discs in tris-buffer solution, that a silica gel formed simultaneously with a calcium phosphate layer, but took longer to develop [6], providing conflicting results with those of other studies which suggested that the calcium phosphate layer formed as a consequence of the silica layer [4, 7, 8]. Further evidence for the presence of a calcium phosphate layer at the interface of bioactive glasses and bone was shown by implanting 16 glasses of the SiO_2–Na_2O–CaO–P_2O_5–Al_2O_3–B_2O_3 system into rabbit tibia, where bone bonding was only found to occur when calcium phosphate build up took place within the silica gel on the glass surface and that the silica gel

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TABLE I Composition of Bioglass 45S5

Compound	Weight percent (wt %)
SiO_2	45.0
Na_2O	24.5
CaO	24.5
P_2O_5	6.0

needed to be sufficiently flexible and hydrated to allow calcium phosphate to accumulate [7]. Other evidence suggests that the bone bonding behavior of bioactive materials is a biological phenomenon rather than being entirely due to the chemical characteristics of the material [10, 37]. It has been reported that on different culture substrates, bone derived cells *in vitro* can generate an afibrillar extracellular matrix rich in calcium, phosphorus and sulphur which is analogous to that of cement lines formed at discontinuities in natural bone [10]. Such matrix formation was not mediated by the culture substrate, but dependent upon cells expressing their osteoblast phenotype, although the material may have played a part by influencing the cell behavior. Indeed osteoblasts grown on Bioglass, compared with non-reactive glass, demonstrated a more typical osteoblast-like morphology, increased proliferation and increased expression of osteoblast-specific proteins [11].

Recently the effect of adding proteins from foetal calf serum (FCS) to SBF on development of the Bioglass surface was studied; using SEM and EDS it was seen that only after 120 h in SBF containing 10% FCS that a calcium phosphate layer formed, whereas without proteins evidence of a calcium phosphate layer was seen after 20 h [13]. These observations were attributed to serum proteins and macromolecules adsorbing onto the Bioglass surface coating the reaction products and inhibiting further ionic exchange so retarding development of the calcium phosphate layer and interfering with nucleation of hydroxyapatite and subsequent crystallization. However, bone bonding to Bioglass has been observed 4 weeks after implantation into rabbit femora [14]. In a second experiment in the same study, Bioglass was implanted into rat femora for 1, 3, 7 and 14 days before histological examination. It was observed that a relatively acellular zone with little surrounding inflammation formed on the Bioglass surface after 1 day and by 7 days new bone formed directly on the glass surface without any distinct boundary where the acellular zone had been. This suggested that the formation of the acellular zone may have been the initial stage of the bone bonding process with subsequent replacement of this zone by bone tissue.

It would therefore seem that *in vivo* the formation on the Bioglass surface of a calcium phosphate rich layer that crystallizes to hydroxyapatite may not be solely due to the chemical characteristics of the material. Proteins and macromolecules in the blood plasma could retard the formation of such layers so that the silica rich layer is dominant on the glass surface and may influence the cellular response and subsequent formation of bone apatite on the implant. For bone bonding to occur, osteoblasts must be able to migrate onto the biomaterial, express a mature phenotype, synthesize and subsequently mineralize osteoid to form the crystalline biological apatite phase of bone. Whether the apatite layer observed at the interface of Bioglass and bone *in vivo* occurs as a consequence of the continued surface reaction of Bioglass or whether it is initiated by cells at the surface expressing the osteoblast phenotype remains unclear. The present study was carried out to examine and compare the responses of bone-derived cells to the surface of either conditioned or untreated Bioglass as a consequence of surface reaction, to determine which aspects of surface development favor cellular colonization.

Materials and methods

Preparation of Bioglass 45S5 samples

Bioglass 45S5 was manufactured (by British Glass Manufacturers Confederation, Sheffield, UK) for use in the present study by mixing reagent grade powders of SiO_2, Na_2O_3, $CaCO_3$ and P_2O_5 for 5 h in a sealed container then heating to 1300 °C in a covered platinum crucible for 20 h. The resulting mixture was cast into a preheated graphite mold to obtain a glass rod and annealed at 450 °C for 4 h before being cut into discs of 15 mm diameter and 1 mm thickness using a low speed diamond saw (Isomet™, Buehler Ltd, UK). The final composition (by wt %) of the Bioglass discs is shown in Table I. Before use in any experiments, the discs were stored in a dessicator to minimize contact with moisture in the atmosphere that may have affected their surface structure. The Bioglass discs were polished, cleaned and sterilized according to the method of Mei *et al*. (1995) [13]. Briefly, after mounting in a polishing machine (DAP-7, Struers, Denmark) and grinding using 1200 grit SiC paper, discs were polished with 6 μm then 1 μm diamond paste, all polishing and grinding materials being obtained from Struers, Denmark. Throughout polishing an ethanol-based lubricant, Dp-blue was used to minimize aqueous contact and to wash away any diamond particles in the final polishing stage. After polishing, the discs were cleaned ultrasonically with xylene for 3 min, degreased in acetone, cleaned with methanol ultrasonically for 10 min and washed twice in 100% ethanol before air drying in a laminar flow cabinet (HF-4, ICN Biomedicals Ltd, UK) and storage in a dessicator. Immediately prior to experiments, Bioglass 45S5 discs were sterilized using dry heat at 160 °C for 1 h.

Surface conditioning of Bioglass 45S5

To allow the surface development of Bioglass discs, immediately after the preparation procedure freshly polished sterile samples were immersed in 7 ml Fitton–Jackson modified BGJb medium (Sigma, UK) supplemented before use (sBGJb) in the following proportions: 100 ml BGJb, 10 ml FCS, 2.5 ml 1 M HEPES and 1 ml 5000IUpenicillin/5 mg/ml streptomycin solution (Sigma, UK). The discs were immersed in sBGJb for 1,2,5,8 or 11 days and kept in an incubator (IG150, Jouan, France) at 37 °C in an atmosphere of 5% CO_2 to allow surface development of the discs.

EDS of Bioglass samples

Samples removed from sBGJb were rinsed in ethanol and air dried in a laminar flow cabinet (HF-4, ICN Biomedicals Ltd, UK) then together with freshly polished, untreated discs, were coated with carbon to a thickness of ~ 40 nm using a carbon coating machine (Polaron TB500, Fisons Instruments, UK). Bioglass 45S5 discs were analyzed using a Jeol JXA-840A scanning electron microscope (Jeol (UK) Ltd) linked with an EDS probe (AN 10 000 X-ray micro-analyzer, Link Systems, UK) at a working distance of 39 mm, a magnification of $\times 2000$ and accelerating voltage of 20 kV. Three discs for each period of development (including previously undeveloped) were studied and five areas were analyzed per disc; one from the center and four equidistant areas around the edge. Each area gave an average elemental composition over 50 μm^2 and was analyzed with a live time of 100 s. An attempt was made to estimate amounts (in wt %) of silicon, calcium, phosphorus and sodium. Unfortunately sodium could not be detected so only the relative values of silicon, calcium and phosphorus were obtained as wt %. Student's *t*-tests (two-sample) were used to calculate significant differences $(p < 0.01)$ between the mean relative proportions of each element at the surface of samples over a period of development for 0–11 days and to examine any differences between the proportions of each element at each time point.

Cell culture

Calvaria were removed from 1- to 2-day-old neonatal albino Wistar rats and the parietal bones isolated, cutting inside the sutures to avoid contamination of the osteoblasts covering the bone surface with the mixture of cell populations present in the sutures. The periosteum was stripped from both the endocranial and exocranial surfaces of the bone and following removal, the periosteal sheets and parietal bones were stored temporarily in separate culture dishes of sBGJb medium.

Periosteal sheets were torn into small fragments and placed onto previously prepared Bioglass discs in 60 mm tissue culture dishes (Corning, USA) containing 7 ml sBGJb medium to establish periosteal cell cultures. These cultures contained a mixed cell population, likely to consist predominantly of fibroblasts with some osteoprogenitor cells and osteoblasts also present.

Osteoblast cultures were established by mincing the parietal bones (stripped of periosteum) using scissors and placing the fragments onto discs in culture dishes containing medium. These cultures were assumed to be predominantly osteoblasts, since cultures established in a similar way from either rat or mouse calvarial cells have been shown to synthesize mineralized bone matrix [15]. All cultures were placed in an incubator (IG150, Jouan, France) at 37 °C in an atmosphere of 5% CO_2 for periods ranging from 4 to 28 days. Medium was replaced every 3–5 days, taking care to minimize disturbance of tissue adhering to the disc surface.

SEM

After surface conditioning Bioglass samples were removed from sBGJb and fixed for 1 h in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Samples were then washed in 0.25 M sucrose in 0.1 M sodium cacodylate buffer for 1 h at room temperature, before dehydration in a graded ethanol series 30–100%, 15 min in each solution. Discs were critically point dried from CO_2 in a Polaron E3100 critical point drier (Agar Scientific, UK) before being sputter coated with gold in a Denton Desk II Sputter Coater (Microfield Scientific Ltd, England). Freshly polished, sterilized unconditioned discs, were also subjected to critical point drying, were immediately coated with gold and all samples were examined using a Jeol 5300 LV scanning electron microscope (Jeol (UK) Ltd).

After use as cell culture substrata, fixation and preparation for SEM was carried out in the manner described previously to study the colonization of periosteal cells and osteoblasts on Bioglass discs. At least three samples for each period of development for both osteoblast and periosteal cells were examined using an accelerating voltage of 20–25 kV.

Results

Effect of immersion in sBGJb on Bioglass surface morphology

Secondary electron SEM examination of freshly polished, cleaned and sterilized Bioglass 45S5 discs revealed a surface that was relatively smooth and flat, compared with the surfaces of pre-treated discs. Polishing scratches and occasional indentations were seen which were likely to be defects caused during the manufacturing of the Bioglass, however the surface appeared not to have reacted as no cracks or reaction layers were visible (Fig. 1(a)).

The Bioglass discs that had been immersed in sBGJb for periods ranging from 1 to 11 days had surfaces with varying degrees of cracking and roughness (Fig. 1(b)–(d)). Whilst the surface of Bioglass immersed for 1 day was still flat, SEM revealed that cracks had formed over much of the surface. Polishing scratches were still visible and SEM at a higher magnification revealed that microcracks of no less than 10 μm in length had formed. After 2 days immersion, it was seen that many of the microcracks had joined to form facets of approximately 10 μm^2 which increased in size with increasing immersion time. After 8 days, the surface of the Bioglass appeared to be formed of at least two layers (Fig. 1(d)) that were possibly separated from the remainder of the Bioglass by the critical point drying process.

Effect of immersion in sBGJb on surface elemental composition

Energy dispersive spectroscopy of Bioglass 45S5 discs which were either untreated or immersed in sBGJb for periods ranging from 1 to 11 days, showed that there were differences in the amount of silicon, calcium and phosphorus measured at the sample surface. Fig. 2 shows the relative proportions (in wt %) of silicon, calcium and phosphorus at the surface of untreated Bioglass (0 days in sBGJb) and samples immersed in sBGJb for 1–11 days. The relative amount of silicon at the surface of Bioglass

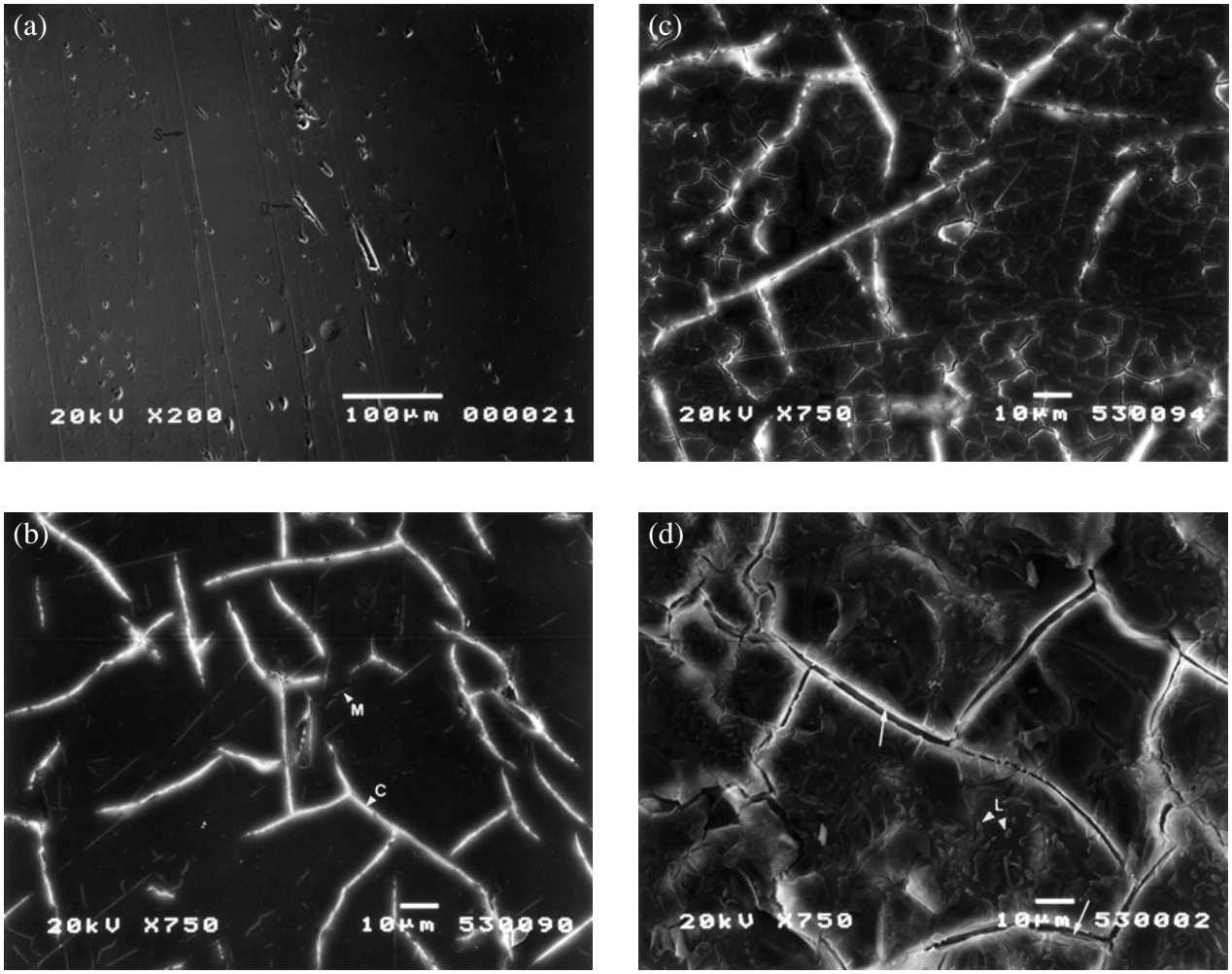


Figure 1 Secondary electron scanning electron micrographs of Bioglass discs immersed in sBGJb for 0–8 days. (a) Freshly polished, cleaned and sterilized sample. The surface was flat with polishing scratches (S) and manufacturing defects (D) clearly seen. Field width 600 µm. (b) 1 day immersion. The formation of microcracks (M) on part of the surface in addition to larger cracks (C) could be seen. Field width 148 µm. (c) 2 days immersion. At high magnification, microcracks were seen to have joined to form facets approximately 10 µm across. Field width 148 µm. (d) 8 days immersion. There appeared to be two layers at the surface (arrowed), visible at certain of the cracks and within the upper layer there was a discontinuous lobular material (L). Field width 148 µm.

increased on immersion in sBGJb for the first day. By 5 days silicon decreased slightly but increased again to reach a peak of 50.2 ± 2.7 wt % at 8 days. After 11 days, the relative proportion of silicon had decreased again, however, there was no statistically significant difference between the amount of silicon at the surface of Bioglass before immersion and 11 days after immersion. The relative proportion of calcium at the surface decreased over the first day of immersion in sBGJb and reached its lowest value of $32.6 \pm 2.0\%$ at 8 days. By 11 days, the relative amount of calcium had increased again although it was significantly less than before immersion ($p < 0.01$). The relative proportion of phosphorus at the Bioglass surface increased over 8 days and had started to fall again by 11 days, although it was still greater than before immersion ($p < 0.01$). Generally, it was shown that silicon made up a significant proportion (in wt %) of the elemental composition at the surface of the discs immersed in sBGJb, even after 11 days.

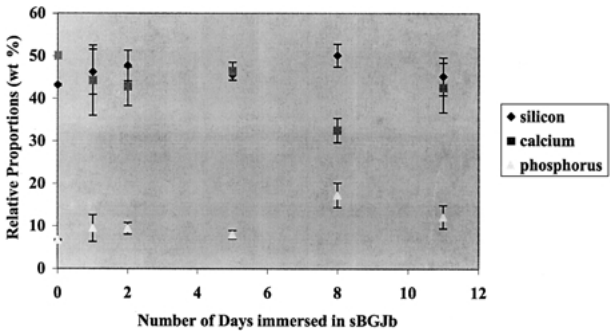


Figure 2 Graph to show the relative change in elemental composition at the surface of Bioglass 45S5 discs before and after immersion in sBGJb for various times as determined using EDS. Error bars represent one standard deviation from the mean.

Cellular colonization of Bioglass samples

Osteoblasts

Osteoblasts would not readily colonize untreated Bioglass samples, since on some samples no cells were seen whilst on others, discrete colonies could be seen to be attached to the Bioglass surface (Fig. 3(a)). The Osteoblasts were flattened and well spread although even after 2 weeks there was little migration with cells only covering small areas of the sample.

All of the pre-treated Bioglass surfaces appeared to

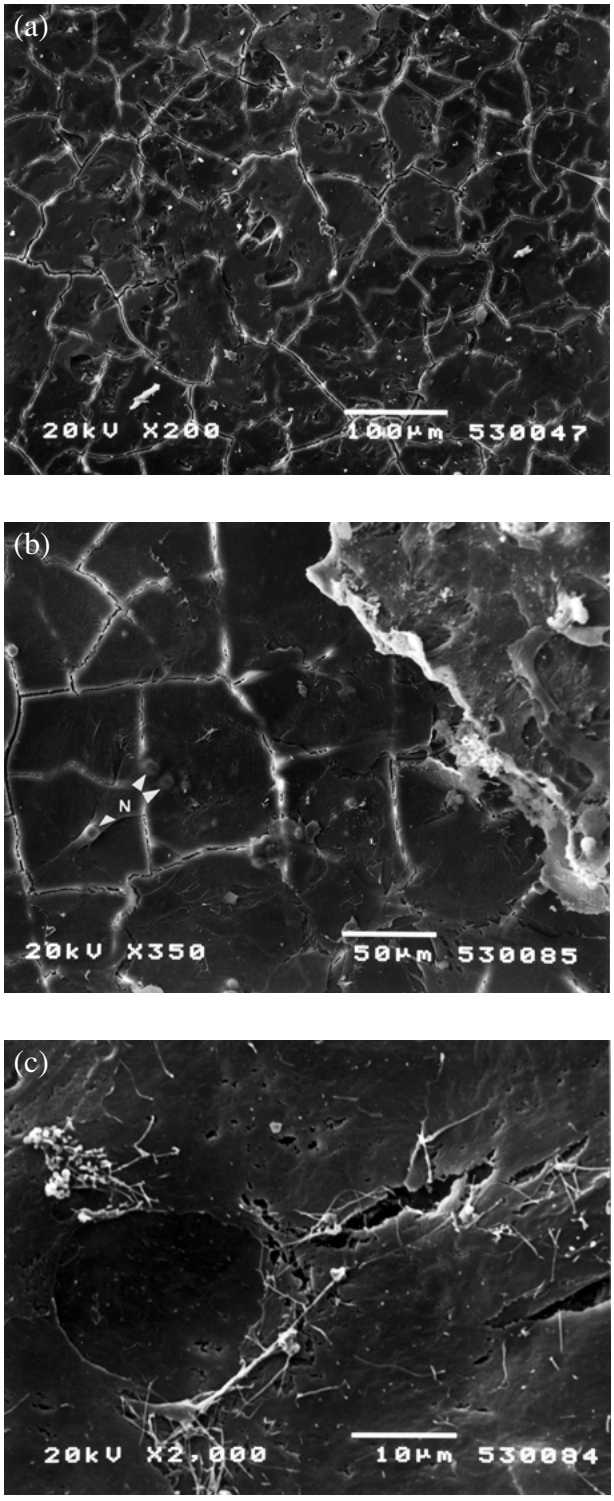


Figure 3 Secondary electron scanning electron micrographs of Bioglass samples used as substrates for osteoblast culture for 2 weeks. (a) Previously polished but untreated sample. Discrete colonies of flattened cells were seen, with individual cells difficult to discern as they were in such close contact with one another. Field width 600 µm. (b) Sample pre-treated for 5 days. Some of the cells which have migrated away from a piece of bone (on the right) appeared to have nodules (arrowed N) immediately beneath their dorsal surface. Field width 323 µm. (c) Sample pre-treated for 5 days, demonstrating fibrous material associated with the cells. Field width 57 µm.

provide a readily colonizable substrate for osteoblasts that migrated away from bone fragments to form continuous sheets on the upper surface film of the Bioglass. After 2 weeks in culture on a Bioglass sample pre-treated for 5 days, more cell migration was observed than seen on untreated samples. The osteoblasts appeared flattened and some had nodules of 10–20 µm diameter visible immediately beneath their dorsal surface (Fig. 3(b)), whilst other cells were associated with fibrous material (Fig. 3(c)), which may have been evidence of extracellular matrix synthesis.

Periosteal cells

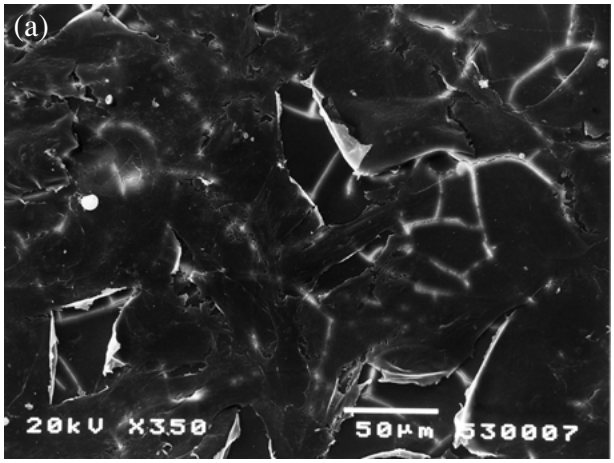
SEM examination revealed that periosteal cells readily colonized untreated Bioglass discs, migrating away from the original piece of tissue to form a well spread, flattened sheet of cells (Fig. 4(a)). After 2 weeks periosteal cells had migrated further than osteoblasts on untreated Bioglass samples. The cells were well spread and in good contact with each other, however, in some areas the cell layer had ruptured caused by the critical point drying process. It was often quite difficult to discern the morphology of individual cells because they were extremely well spread and in very close contact with each other.

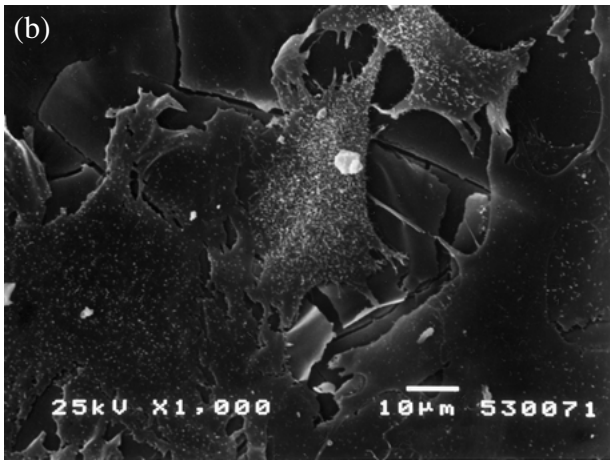
SEM of Bioglass samples pre-treated in sBGJb for 1–11 days revealed that these surfaces all provided suitable substrates for colonization by periosteal cells. On Bioglass discs immersed for 1 day in sBGJb before use as periosteal cell culture substrates for 2 weeks, it was observed that cells were directly attached to the upper surface film of the Bioglass (Fig. 4(b)). The cells were growing in multilayers, appeared well spread with a polygonal morphology and dorsal membrane ruffles, they were in close contact with each other and the surface film.

High magnification of cells cultured for 2 weeks on Bioglass that was pre-treated for 8 days in sBGJb showed cells to be colonizing a ruptured upper layer, on top of which were globular accretions, possibly associated with the cells (Fig. 4(c)) although globules were also seen on top of the reacted surface on other samples in areas where no cells were present. These SEM observations suggest that the periosteum-derived cells in the present study may have been more able than osteoblasts to colonize Bioglass surfaces and that the polygonal morphology of periosteum-derived cells on the Bioglass appeared similar to that of osteoblasts. Periosteal cells were found in layers on the Bioglass samples more often than osteoblasts were and would readily proliferate onto discs not pre-treated in sBGJb whereas osteoblast migration onto these surfaces was considerably less.

Discussion

The formation of the irregular cracks on the surface of the Bioglass samples during surface conditioning in sBGJb for periods ranging from 1 to 11 days and subsequent surface development during use as culture substrates, was attributed to the dissolution of ions from the glass into the culture medium. When compared with samples immersed in sBGJb for longer periods of time, the samples immersed in sBGJb for short periods (i.e. up to 2 days), exhibited notably less crack formation. This was possibly a reflection of a shorter reaction time where less ionic dissolution had occurred. It has previously been shown that immersing Bioglass 45S5 in culture medium (α-MEM containing 5% FCS) for periods





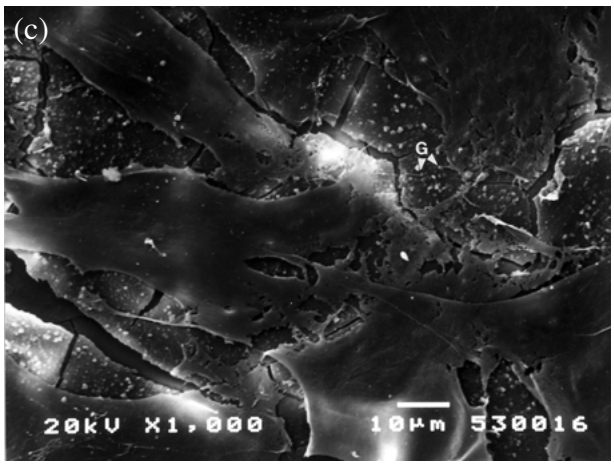


Figure 4 Secondary electron scanning electron micrographs of Bioglass samples used as substrates for periosteal cell culture for 2 weeks. (a) Previously polished but untreated sample. A layer of well spread, flattened cells covered the Bioglass surface. In some areas the cell layer ruptured, possibly as a result of critical point drying and the cracked Bioglass surface was revealed beneath. Field width 323 µm. (b) Sample pre-treated for 1 day. Polygonal shaped cells with dorsal membrane ruffles were seen to be colonizing the upper surface layer of the Bioglass. Field width 114 µm. (c) Sample pre-treated for 8 days. Cells were seen to be colonizing the ruptured upper surface layer of the Bioglass, on top of which were globular accretions (arrowed G), intimately associated with cell extensions. Field width 114 µm.

ranging from 2 to 16 days caused irregular cracking at the surface and that dehydration during critical point drying was suggested as cause of further shrinkage of the reacted layer [11, 16]. It has been shown previously that amino acids from α-MEM were incorporated into the growing Ca, P-rich surface layer of Bioglass subsequently causing instability of this layer, demonstrated by a lower resistance to stresses that occurred during the critical point drying process [17].

The present study demonstrated that although calcium and phosphorus had started to accumulate by 11 days, the relative proportion of silicon at the Bioglass surface was still extremely significant (at least 45%), since silicon levels did not dimmish over the 11 days in sBGJb. The EDS employed in the present study only analyzed the Bioglass to a depth of 1–3 µm although it has been shown previously using EDS examination of cross sections of Bioglass discs immersed in sBGJb for 40 days, that the total reaction layer was 27 µm thick [18]. Mei [18] found that the uppermost silica-rich layer with decreasing amounts of Ca and P with depth, had a thickness of 3 µm, therefore the elemental analysis in the present study would appear to represent only the formation of this superficial layer. Indeed, previous studies have demonstrated that the inclusion of proteins in the immersion solution significantly retards the development of the Bioglass surface [11, 19].

Studies in which Bioglass was soaked in either SBF or tris-buffered solutions without the presence of proteins demonstrated that crystallization of hydroxyapatite occurs on Bioglass surfaces after just a few hours [6, 20], whilst the inclusion of proteins in SBF has been shown to retard crystallization of calcium phosphate on the developing Bioglass surface [13, 22]. Using a pH-stat system, maintaining a constant pH 7.4, that serum proteins adsorbing to growing hydroxyapatite crystals is one mechanism of crystal growth inhibition and that albumin, the most abundant serum protein, is an important mediator of this process [21]. However, in the present study osteoblasts colonized the discs and could have contributed to the apatite formation by the secretion of enzymes (such as ATPase) that hydrolyze inhibitors of calcification and/or secretion of other substances (such as bone sialoprotein (BSP)) providing nucleation sites for hydroxyapatite crystal growth. Indeed it has been shown previously using Northern hybridization, that BSP was synthesized by rat marrow stromal cells that had differentiated into osteoblasts and produced bone-like mineralized tissue on a bioactive glass ceramic *in vitro* [23].

A possible explanation for the consistently poor osteoblast colonization of previously undeveloped, freshly polished Bioglass discs in the present study is that immediately on immersion the surface was too reactive and therefore the surface free energy may have been too high to favor osteoblast colonization. Indeed, Vrouwenvelder *et al*. [16] noted foetal rat osteoblasts with a ''stand-off'' morphology after 2 days culture on Bioglass 45S5 and suggested that the high initial free surface energy was the reason for the observed morphology [16]. It could be that it was only after some time when the surface had been modified that cells would migrate onto the discs and that this was the reason for the low number of cells actually seen to colonize the discs. In other studies it was observed that after 6 days, osteoblasts formed a confluent dense monolayer on the Bioglass surface and had started to synthesize type I collagen after 12 days [11, 16]. These results are in stark

contrast to those in the present study in which cells on previously untreated Bioglass surfaces were far from confluent after 2 weeks in culture and may be explained by different experimental methods when establishing osteoblast cultures on the Bioglass surfaces. In the present study, cultures were established without the use of proteolytic enzymes, whereas in other studies collagenase has been utilized to isolate bone-derived cells [11, 16]. The exact effects of such enzymes on cells are not fully understood, however they are known to affect cell behavior in their use during isolation procedures [24]. Proteolytic enzymes have been shown to alter both cell adhesive behavior and morphology by the removal of cell membrane glycoproteins involved in cell adhesion and causing disaggregation of intracellular microtubules involved in the determination of cell shape [25]. Proteolytic enzymes are likely therefore to have significant effects on the properties exhibited by the cells when cultured *in vitro* and therefore not give an accurate representation of how cells may behave on a foreign substrate *in vivo*.

It has previously been shown that protein adsorption to Bioglass differs according to its surface development [26]. These differences in protein adsorption on Bioglass are very likely to affect subsequent cell attachment since it is via serum proteins that cells attach to material surfaces [27]. In the present study, therefore, it could have been that the proteins adsorbing to unreacted Bioglass did not favor osteoblast attachment and so colonization of these surfaces was less than that observed on pre-treated discs. Indeed, improved ROS 17/2.8 cell adhesion on surface reacted Bioglass compared with that on unreacted Bioglass, has been shown to result from enhanced cell receptor–fibronectin interactions, as a consequence of substrate-dependent conformational changes in the adsorbed fibronectin [36].

In previous studies, the surface of Bioglass was modified in different ways before seeding with rat calvarial osteoblasts to examine the effects of various surface structures on the expression of the osteoblast phenotype [28]. Immersion of Bioglass in tris-buffer for 48 h led to the formation of a calcium phosphate-rich layer which provided sites for the adsorption of serum factors when samples were subsequently immersed in tissue culture medium. The concentration of these serum factors at the glass surface was suggested to enhance the expression of the osteoblast phenotype.

In the present study, EDS revealed that silicon was present in significant amounts at the Bioglass surface for at least 11 days in sBGJb and all the pre-treatment times of Bioglass appeared to provide a substrate readily colonized by osteoblasts. It may be that certain cell adhesion proteins from the sBGJb adsorbed onto the silica-rich surface and promoted cell attachment via interaction with specific binding sites on osteoblast membranes. It has been shown that silicon released as silicic acid from Zeolite A (an aluminosilicate polymer), stimulated proliferation in human osteoblast-like cells *in vitro* [29], therefore the biologically active silanol groups present on the silica-rich surface could have stimulated proliferation of osteoblasts on the Bioglass surface in the present study. Furthermore, a combination of silicon and calcium from Bioglass that had leached into the culture media of periodontal ligament fibroblasts was shown to increase the number of mineralized nodules composed of phosphorus and calcium observed on these cells [30]. The increased activity of alkaline phosphatase measured in the supernatant from such cultures led to the suggestion that since the enzyme is one of the markers of the osteoblast phenotype, the calcium and silicon from the Bioglass had an effect upon the differentiation of these cells *in vitro*. Although in the present study, the expression of the osteoblast phenotype was not examined on such cells colonizing Bioglass, there was possible evidence of extracellular matrix synthesis and development of nodules on the membranes of cells.

The present study showed that periosteal cells colonized untreated Bioglass *in vitro* more readily than osteoblasts as cells derived from periosteal tissue readily migrated onto untreated, freshly polished Bioglass surfaces whereas only few discrete colonies of osteoblasts were seen after 2 weeks. The periosteum-derived cells formed a flattened ''sheet'' that appeared continuous with the upper surface reaction film seen after a culture period of 2 weeks, suggesting that the surface of the Bioglass was suitable for colonization by these periosteal cells. This implied that either the adhesion of periosteal cells to Bioglass was mediated via different proteins than those mediating osteoblast attachment, or that periosteal cells were less sensitive to the reactive Bioglass surface. Indeed, it has been shown previously that compared with primary osteoblasts, periosteal cells exhibited an apparent decreased sensitivity to elements released from amalgam alloys *in vitro* [31].

Other studies cultured fibroblasts on Bioglass 45S5 with subsequent observations, which were strikingly different from those of the current study. It was noted that after 7 days in culture fibroblasts had only sparsely colonized the Bioglass surface, were smaller and became long and slender with radially spread long pseudopodia compared with the same cells grown on inert silica glass used as a control [32]. Such shape changes were suggested to indicate that the cell cycle was arrested at the S phase. The growth rate of these fibroblasts on Bioglass was markedly lower than seen on controls, which was suggested to be due to the release of alkali ions from Bioglass, increasing the pH of the culture medium to a level unsuitable for optimal growth. Experimental differences could explain the modified responses of fibroblasts on the Bioglass observed in the present study. For example, the samples of Bioglass 45S5 were prepared in a different way prior to use in culture which could have influenced the surface microstructure of Bioglass and affected subsequent surface development *in vitro*. Fibroblasts in the present study were primary cells that had migrated away from pieces of periosteal tissue and so the cell population would also include osteoprogenitor cells and some osteoblasts. It is conceivable that these primary cells would behave differently to those of the fibroblast-derived cell line (L cells) used in the previous study as cell lines are known to have the potential to divert from their original phenotype and lose tissue specific markers [33].

As observed with osteoblasts, all pre-treatment times of Bioglass in sBGJb in the present study resulted in Bioglass surface structures suitable for periosteal cell

colonization with the period of immersion in sBGJb appearing to be unimportant from 1 to 11 days. There was more migration of periosteal cells over the Bioglass surface than observed with osteoblasts, although the extent of this was not quantified. Globules that appeared to be associated with the cells were seen on the Bioglass surface and it is possible that they were synthesized by the cells or that these globules were evidence of further development of the Bioglass surface. It has previously been found using scanning and transmission electron microscopy that rat bone marrow stromal cells on various substrates form multilayers prior to synthesis of mineralized bone nodules [10] and, in the present study, periosteal cells were also observed to form multilayers on the Bioglass surfaces. Furthermore, the previous study showed that an early stage of extracellular matrix synthesis involved the accumulation of globular accretions over the substrate surface which on closer examination revealed that the individual processes of cells terminated with such accretions. The production of such globules was dependent on cells expressing the osteoblast phenotype, as globules were only produced in significant amounts when the cultures were supplemented with 10^{-8} M dexamethasone, 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate, independently of whether or not the surface was treated to favor cell colonization. It could be, therefore, that the accretions on the Bioglass surface in the present study were evidence of early extracellular matrix synthesis by the periosteally derived cells colonizing the Bioglass surface, indeed it has been demonstrated previously that Bioglass influences the differentiation of osteoprogenitor cells [34].

Conclusions

It can be concluded that the development of the silica-rich layer formed on the Bioglass surface is important in the response of bone-derived cells to Bioglass since silicon is present in relatively high amounts for a considerable time after exposure of the material to protein-containing solution.

Initially on immersion, the surface of Bioglass was not suitable for optimal colonization by osteoblasts whereas periosteal-derived cells, representing an osteoprogenitor cell population would readily colonize the surface. The results of the present study indicate a need to closely examine the effects of silicon upon the behavior of bone-derived cells, particularly with regards to cell differentiation along the osteoblast lineage.

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